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Characterization Of Human T-Bet-Expressing B Lymphocytes And Their Role In The Hiv Immune Response

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Abstract

Humoral immunity is critical for the prevention and control of viral infections, yet the specific B cells and mechanisms regulating antiviral responses in humans remain poorly defined. The Th1-associated transcription factor T-bet coordinates intracellular pathogen immune responses, and recent murine studies identified a T-bet-expressing B cell subset that mediates humoral antiviral immunity, but an analogous cell population has not been identified in humans. In this study, we sought to investigate the role of T-bet-expressing B cells during human viral infections. We identified T-bet expression within the memory B cell compartment of healthy individuals and described a relationship between the transcription factor and IgG1 and IgG3, two antiviral antibody isotypes. The T-bet+ B cell population was comprised of two discrete subsets: T-bet low resting memory cells and a highly activated, transcriptionally distinct T-bet high subset displaying an atypical memory phenotype. The T-bet high cell population transiently expanded in blood following vaccination with yellow fever or vaccinia virus; however, these cells were induced and maintained at an elevated frequency by chronic HIV viremia and were associated with increased expression and secretion of IgG1 and IgG3. The HIV gp140-specific response was maintained almost entirely by T-bet+ memory B cells in both viremic and aviremic donors. Together, our findings identify T-bet as a critical regulator of humoral antiviral immunity in humans and suggest T-bet+ B cells specifically mediate the humoral immune responses to live viral vaccines and HIV.

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CHARACTERIZATION OF HUMAN T-BET-EXPRESSING B LYMPHOCYTES AND THEIR
ROLE IN THE HIV IMMUNE RESPONSE

James J. Knox

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DEDICATION

I dedicate this dissertation to my parents, Jim and Marybeth, for instilling in my brothers and me the value of hard work and providing love and support for as long as I can remember.

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ABSTRACT

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Humoral immunity is critical for the prevention and control of viral infections, yet the specific B cells and mechanisms regulating antiviral responses in humans remain poorly defined. The Th1-associated transcription factor T-bet coordinates intracellular pathogen immune responses, and recent murine studies identified a T-bet-expressing B cell subset that mediates humoral antiviral immunity, but an analogous cell population has not been identified in humans. In this study, we sought to investigate the role of T-bet-expressing B cells during human viral infections. We identified T-bet expression within the memory B cell compartment of healthy individuals and described a relationship between the transcription factor and IgG1 and IgG3, two antiviral antibody isotypes. The T-bet⁺ B cell population was comprised of two discrete subsets: T-bet^{low} resting memory cells and a highly activated, transcriptionally distinct T-bet^{high} subset displaying an atypical memory phenotype. The T-bet^{high} cell population transiently expanded in blood following vaccination with yellow fever or vaccinia virus; however, these cells were induced and maintained at an elevated frequency by chronic HIV viremia and were associated with increased expression and secretion of IgG1 and IgG3. The HIV gp140-specific response was maintained almost entirely by T-bet⁺ memory B cells in both viremic and aviremic donors. Together, our findings identify T-bet as a critical regulator of humoral antiviral immunity in humans and suggest T-bet⁺ B cells specifically mediate the humoral immune responses to live viral vaccines and HIV.

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CHAPTER 1

INTRODUCTION

1.1 B cells and immunological memory: an introduction

B lymphocytes, or B cells, were first described as a separate lymphocyte lineage from T cells in 1965 by Cooper et al., named as such for their bursa-dependent, versus thymus-dependent, development in birds (1). A number of seminal studies established B cells as the mediators of humoral immunity that give rise to plasma cells and ultimately regulate the production of antibodies, soluble γ -globulin proteins that recognize pathogens (2). B cells and antibodies are necessary for successful immune responses to various viruses, bacteria, fungi, and parasites, mediating pathogen clearance directly by neutralization or in concert with the innate and adaptive immune systems (3). The ability to harness humoral immunity through vaccination stands as perhaps the greatest public health achievement, as vaccines have greatly reduced the incidence of numerous infectious diseases and have eradicated smallpox (4). Notably, nearly all licensed vaccines are thought to function primarily through the elicitation of protective antibodies and B cell memory (4), highlighting the powerful capacity of the humoral immune system to afford protection against invading pathogens.

One of the hallmarks of adaptive immunity is its ability to establish long-lived memory cells capable of recognizing previously encountered antigens and inducing a focused immunological response. Upon activation by their cognate antigens, mature T and B cells give rise to a suite of antigen-experienced progeny, including effector cells that mediate the acute response and long-lived memory cells that persist as sentinels prepared for re-infection (5). Upon antigen re-encounter, memory B cells fulfill several functions that contribute to the immune response: they are poised to quickly differentiate into antibody-secreting cells, but they also can seed new germinal center reactions to further mature their B cell receptor via hypermutation and isotype switching, processes that improve antigen binding affinity and increase the diversity of

antibody effector functions (6). An improved understanding of B cell biology during the generation of humoral immune responses will inform vaccine development and therapeutic interventions to strengthen immunity.

1.2 Generation of humoral immunity

Early B cell Development

The lineages of all antigen-specific B cells can be traced back to the bone marrow. B cell development in adults begins in the bone marrow, a niche with specific growth factors such as IL-7 and other signals necessary to guide the differentiation of hematopoietic stem cells through multipotent myeloid/lymphoid progenitor and common lymphoid progenitor stages, and finally into the B cell lineage (7). Early B cell progenitors are restricted to the B cell lineage upon upregulation of Early B cell Factor 1 (EBF1) and Paired Box 5 (Pax5), two transcription factors that function within a broader transcriptional network to regulate the B cell developmental process and its stepwise expression and rearrangement of the B cell receptor (BCR) genes (8, 9). B cell precursors complete rearrangement of their V_H , D_H , and J_H segments to form the BCR heavy (IgH) chain and express it on the surface, where it can signal to promote allelic exclusion and, in complex with the surrogate light chain as the pre-BCR, confirm the heavy chain's ability to complex with a light chain (10, 11). Surviving pre-B cells rearrange the V_L and J_L segments to create the light (IgL) chain and finally join the heavy and light chains together to form a functional BCR with an IgM constant region (12).

Newly formed immature B cells that are sufficiently non-self-reactive will egress from the bone marrow into the periphery, where they are termed 'transitional' B cells (12, 13). Transitional B cells undergo additional selection and maturation processes in secondary lymphoid tissue sites such as the spleen, where their survival is dependent upon both BCR signaling strength and concentrations of B lymphocyte stimulator (BLyS), a B cell survival factor. Appropriate signal

combinations allow transitional B cells to seed the mature naïve B cell compartment (14, 15). The resulting mature B cell pool demonstrates extraordinarily diverse antigen recognition potential capable of engaging nearly any incoming pathogen or antigen. Naïve B cells, which account for nearly half of all B cells in adults, therefore function as a reservoir of diverse BCR specificities waiting to encounter their cognate antigen (11).

The initiation of humoral immune responses

In lymphoid tissues, naïve B cells recognize and bind their cognate antigen using surface-expressed BCR. Antigen binding transduces activation signals via BCR-associated Ig α and Ig β molecules containing immunoreceptor tyrosine-based activation motifs (ITAMs) and is enhanced by concurrent activation of a co-receptor complex consisting of CD19, CD21, and CD81 (16). Phosphorylation of ITAMs by Src-family tyrosine kinases recruits molecules such as spleen tyrosine kinase, phospholipase C γ 2, and phosphoinositide 3-kinase to initiate signaling cascades resulting in calcium mobilization, transcriptional activation, cytoskeletal rearrangement, and internalization of BCR-antigen complexes (17). *In vivo*, this activation process often occurs in the context of B cell-follicular dendritic cell (FDC) interactions and is facilitated by the formation of an immunological synapse, a specialized structure concentrating multiple BCR-antigen and signaling molecule complexes at the interface between the two cells (16).

B cells can subsequently undergo T cell-independent (TI) development, which results in production of low-affinity plasmablasts and small numbers of memory B cells, or begin the T cell-dependent (TD) pathway. The TD activation process continues as B cells process and subsequently present internalized antigens on major histocompatibility complex (MHC) molecules to cognate T cells at sites such as the border of the T cell and B cell zones or the interfollicular zone (17, 18). Here, B cells begin extended interactions with recently activated antigen-specific CD4⁺ T cells and proliferate (19). Several fates are possible following T cell interactions: 1) a subset of activated B cells further migrate into extrafollicular regions and give rise to unswitched memory B cells or short-lived plasmablasts that produce low-affinity antibodies, while 2)

additional B cells begin the germinal center maturation pathway and eventually give rise to high affinity memory B cells and plasma cells (19).

Both activated memory B cells and CD4⁺ T cells destined to generate a germinal center reaction undergo a differentiation process regulated in part by the transcription factor B cell lymphoma protein 6 (Bcl-6; (20)). Alterations in homing receptor expression, including upregulation of CXCR5 and downregulation of CCR7, permit the migration of these cells, now referred to as germinal center (GC) B cells and T follicular helper (Tfh) cells, into the center of the B cell follicle (21, 22). B cells congregate amongst follicular dendritic cells (FDCs), an antigen-presenting population, proliferate, and eventually form a large structure comprised of light and dark zones (23). In the light zone, B cells compete for Tfh cell help: the process is dependent upon BCR affinity, as high affinity B cells bind and uptake more antigen from FDCs, and internalized antigens are processed and presented via MHC-II molecules to Tfh cells (24). Increased antigen presentation correlates with greater Tfh help signaling in the form of cell-to-cell contact (i.e. CD40-CD40L interactions) and helper cytokines (i.e. IL-21) that promotes B cell survival (19). B cells receiving insufficient Tfh help undergo apoptosis, while B cell clones obtaining Tfh help migrate into the dark zone, proliferate extensively, and simultaneously introduce point mutations into the antigen binding regions of their BCR (19, 25). B cells can migrate back into the light zone for additional Tfh help, and these iterative cycles of competition, proliferation, and mutation improve BCR affinity for the antigen and ultimately select for high-affinity B cell clones (25). Isotype switching, the process of changing the expressed antibody class from IgM to alternative isotypes with diverse functions, also takes place within the light zone (26). Upon exiting the germinal center, high affinity GC-produced B cells can differentiate into memory B cells or antibody-secreting cells (5).

1.3 Antibodies and their function

Antibody-secreting cell fate decisions

Plasma cells and their immature precursors plasmablasts (collectively termed antibody secreting cells (ASCs)) represent the terminal differentiation state of the B cell lineage and function to constitutively produce and secrete antibodies (27). During early immune responses, these cells derive from activated B cells via a GC-independent mechanism to generate low-affinity antibodies as a first line of defense. As immune responses develop, longer-lived ASCs with high-affinity antibodies are generated by T-dependent germinal center reactions (27, 28). In both cases, adoption of the ASC fate requires partial suppression of B cell identity, which is achieved by transcriptional repression of Bcl-6 and Pax5 by the transcription factor PR domain zinc finger protein 1 (PRDM1, or BLIMP1; (29, 30)). Together with BLIMP1, the transcription factors interferon regulatory factor 4 (IRF4) and X box binding protein 1 (XBP1) further promote the ASC developmental program and drive plasmablast differentiation (31-33). Plasma cells migrate to specific tissues such as bone marrow and gut-associated lymphoid tissue (GALT), where they can persist for decades and continue to secrete protective antibody (34, 35).

Antibody structure and function

Antibodies (also known as immunoglobulins, or Ig) are globular proteins that exist either in a cell membrane-expressed form as the BCR or in a soluble form within serum and at sites of pathogen encounter such as mucosal surfaces. Antibodies are comprised of two identical sets of heavy chains and light chains connected by disulfide bonds to form a distinctive Y-shape, with an antigen binding region (F_{ab}) at the two tips and a constant region (F_c) at the base that interacts with immune system components (3). The F_{ab} region contains variable domains with complementarity-determining regions (CDRs) that directly interact with antigen, can be highly mutated, and are interspersed between framework regions (FRs) that maintain domain structure (3). Conversely, the F_c region lacks this mutational diversity but exists in several different isotypes

that inform antibody function: IgM, IgD, IgA (two subtypes), IgG (four subtypes), and IgE . Modifications such as glycosylation can further influence the functionality of each isotype by affecting F_c receptor interactions and antibody half-life (36).

The utility of antibodies is based upon their ability to diversify their antigen binding domains and switch their isotype classes, two processes that require activation induced cytidine deaminase (AID; (37)). Functionally, this B cell-specific enzyme converts deoxycytidine (dC) to deoxyuracil (dU) at targeted DNA sites, leading to induction of point mutations that facilitate somatic hypermutation or double strand breaks that enable class switch recombination (37, 38). AID expression is tightly regulated transcriptionally, post-transcriptionally, and at the protein level by a number of factors in an effort to minimize AID activity at off-target genetic loci that could cause cell transformation (38). Within the B cell compartment, AID is primarily expressed within GC B cells but is upregulated following B cell activation can also be expressed during extrafollicular B cell development (39). AID expression is critical for the development of high affinity antibodies and memory B cells during immune responses (24, 40).

Antibodies function to mediate pathogen clearance in several ways that are directly dependent upon AID-regulated somatic hypermutation and class switching. Antibodies can directly neutralize pathogens by interfering with processes necessary for infection and pathogenesis (e.g. preventing interaction between virions and cell surface receptors necessary for viral entry (3)); neutralization is improved with affinity maturation and is generally independent of the F_c region (41). Antibodies can also act as intermediaries via opsonization, the marking of infected cells or pathogens for death or immune clearance. This process is dependent upon the F_c region's ability to activate the complement system and engage phagocytic cells and natural killer cells that express F_c receptors (3, 42, 43). Different types of pathogens elicit specific antibody isotypes to mediate relevant effector functions; human antibody isotype diversity will be discussed below.

Antibody isotypes

IgM is the first isotype B cells express during development (17). IgM is generally of low affinity, demonstrating reduced rates of somatic hypermutation and increased polyreactivity compared to other isotypes. To compensate for this, IgM is secreted in a pentameric complex joined together by disulfide bonds to enable high avidity interactions with antigens (3). IgM is the first serum antibody produced during an acute infection before germinal centers are fully established; however, significantly mutated IgM can be produced by germinal center-derived memory B cells and plasma cells later in the response (44). Functionally, IgM antibody can opsonize antigens and efficiently activates the complement system, and evidence in humans suggests a niche for IgM⁺ memory B cells in seeding secondary germinal center reactions at the expense of undergoing plasma cell differentiation upon antigen re-encounter (45). Thus, IgM plays an important role at multiple points during the course of both primary and secondary infections.

The biological function of IgD remains unclear, as it is rarely secreted into the serum. During development, IgD is first detected on naïve B cells, where it is co-expressed with IgM (17). A recent study suggests IgD may chiefly function in a signaling capacity as a BCR on naïve cells, as it recognizes multivalent antigens more efficiently than the IgM isotype (46). However, a number of observations suggest wider roles for this antibody isotype: most IgM-expressing memory cells continue to co-express IgD (6), class-switched IgD⁺ memory B cells ("IgD-only") can be found in peripheral blood at low frequencies (47), IgD⁺ plasma cells have been identified in tonsils and the human upper airway (48), and secreted IgD has been shown to bind and activate basophils (48).

IgA antibodies are critical components of mucosal immunity that can be secreted in a dimeric structure. While detectable in serum at a titer equal to or greater than IgM, IgA is the most prevalent isotype at mucosal surfaces and in secretions (3). The IgA class is comprised of IgA1 and IgA2 subtypes that differ structurally in their hinge regions, with IgA1 being prevalent in serum and IgA2 in secretions. Plasma cells in GALT constitutively produce IgA that can be

transported across intestinal epithelial cells via the polymeric Ig receptor into the lumen, where it protects the epithelium from infection and regulates commensal bacteria populations (49).

Functionally, IgA mediates neutralization but does not efficiently activate complement or opsonize antigens (3).

IgE mediates immune responses to helminths and plays a role in allergic responses.

While serum IgE levels and frequencies of IgE⁺ memory B cells in peripheral blood are low, IgE is a potent activator of mast cells and basophils, which express the high affinity FcεRI (50). The interaction of FcεRI-bound IgE with antigen leads to degranulation and release of inflammatory mediators from mast cells that exacerbate allergic reactions (50). IgE also binds to a low affinity FcεRII receptor expressed on B cells, through which it regulates antibody production (51).

IgG class antibodies are the most prevalent in human serum, in part due to their long half life. There are four subtypes, named in order of abundance (IgG1>IgG2, etc.), with distinct structural and functional profiles (52). IgG1 and IgG3 are induced by protein antigens, particularly in the context of a TD response, and are notable for their ability to engage effector cells: they efficiently activate complement and bind to all three classes of FcγRs, although IgG3 generally has higher affinity (3, 6, 52). As such, IgG1 and IgG3 isotypes are critical for antiviral responses and are particularly inflammatory (52). IgG3 is produced during the early immune response to viral infections and is eventually overshadowed by IgG1 as the response progresses (53, 54); the gradual loss of IgG3 is based upon the structure of the F_c region locus (i.e. IgG3⁺ memory cells can switch to become IgG1⁺, but not vice versa) and IgG3's reduced half-life compared to other IgG isotypes (IgG1 outcompetes IgG3 for neonatal Fc receptor-mediated recycling (53)). In contrast, IgG2 is critical for polysaccharide antigen responses, as evidenced by the association between IgG2 deficiency and increased susceptibility to encapsulated bacteria infections. IgG2 is often produced during TI immune responses, but many IgG2⁺ memory B cells show evidence of a germinal center origin (55, 56). IgG4 is a rare isotype that can be induced in a number of settings, including polysaccharide antigen responses and settings of allergy (52). IgG2 and IgG4 poorly

activate complement and have low affinity for most Fc γ Rs; as such, they are relatively inefficient opsonizers compared to the IgG1 and IgG3 isotypes (3).

1.4 Memory B cells

Memory B cell fate decisions

Like ASCs, memory B cells can also differentiate from T cell-independent (TI) or T cell-dependent (TD) immune responses, having either extrafollicular or germinal center origins in the latter case (5). However, in contrast to ASCs, the molecular mechanisms regulating adoption of memory B cell fate after germinal center exit are largely unclear. While a master transcription factor akin to BLIMP-1 has not been identified, the transcriptional regulator BTB Domain and CNC Homolog 2 (Bach2) may be important for memory differentiation (57, 58). Survival of the germinal center reaction in the absence of ASC-inducing signals has also been proposed to inherently promote assumption of the memory fate (59), and events predating this fate choice such as isotype class switching may even affect memory differentiation (60). Regardless, the memory B cell compartment functions as a reservoir of cells primed to respond to pathogen re-encounter, orchestrating a rapid secondary response culminating in antibody production to contain and clear the pathogen before widespread pathology can occur.

The memory B cell compartment is exceptionally diverse, a property reflected not only in its expression of the different antibody isotypes, but also in the various origins, functions, and phenotypes of its members. Despite this diversity, these cells are reliably identified by the presence of antibody somatic hypermutation, isotype switching, and phenotypic signatures associated with a post-germinal center state (6). In this dissertation, “memory B cells” will broadly refer to the antigen-experienced B cells produced by immune responses that maintain the B cell phenotype (i.e. excluding ASCs): this group includes both long-lived, quiescent cells and those actively participating in the immune response.

Memory B cell functions

Memory B cells fulfill a number of roles that both support developing immune responses and contribute to pathogen clearance. The ability to readily differentiate into plasmablasts producing high-affinity antibodies separates memory from naïve B cells and is perhaps the most important function of B cell memory (6). Memory B cells can also re-enter and form new germinal center reactions upon pathogen re-encounter, a process that diversifies the antigen-specific antibody pool by further fine-tuning antibody affinity and broadening the isotype repertoire (61). Via these mechanisms, memory B cells can increase the baseline volume of antibody produced by existing plasma cells from the primary response and diversify the F_{ab} region to facilitate responses to mutating pathogens.

Memory B cells also contribute to the immune response with antibody-independent functions. B cells highly express MHC-II molecules, which they use to present processed antigens to $CD4^+$ T cells (16). Antigen presentation is an important feature of GC B cells that facilitates interactions with Tfh cells in germinal centers to promote mutual activation, proliferation, and differentiation (62), and subsets of post-germinal center B cells appear to be efficient antigen presenters in specific tissue sites such as the mouse spleen (63). Memory B cells also produce cytokines that shape the immune response: B cells can produce IL-12 and IFN γ , which promote Th1-type (intracellular pathogen) immune responses, or IL-4 and IL-13, which promote Th2-type (extracellular parasite) immunity (64, 65). B cells produce additional cytokines such as tumor necrosis factor and IL-6, and a subset of B cells, termed regulatory B cells, produce the cytokines IL-10 and/or transforming growth factor beta to suppress immune responses (66).

Human memory B cell phenotypes

Memory B cells are derived from naïve B cells following antigen-induced activation, leading to the accumulation of mutations in antibody variable domains and (in some cases)

switching from the IgM⁺IgD⁺ isotype. Memory B cells were originally identified by the IgD⁻ phenotype (67) until the discovery of somatically mutated IgD⁺ B cells suggested that isotype expression was insufficient to delineate memory (68, 69). A correlation between somatic mutation and CD27 expression was concurrently described, identifying CD27, a tumor necrosis factor receptor family member, as a B cell memory marker in the spleen and peripheral blood (68, 69). Appropriately, CD27⁺ B cells were found to be larger, contained increased cytoplasm, and readily differentiated into class-switched ASCs following antigen and/or cytokine stimulation (70).

Subsequent studies would identify memory B cell subsets that do not express CD27. First, a peculiar CD27⁻ population expressing Fc receptor homologue 4 (FCHR4, now referred to as FcRL4) was described in human tonsil, with comparable somatic mutation and activation marker expression to classical tonsillar memory B cells (71). CD27⁻IgG⁺ populations were also identified in the peripheral blood, comprising 25% of total IgG⁺ B cells (72); these CD27⁻IgG⁺ cells phenotypically and morphologically resembled their CD27⁺ counterparts, albeit with slightly reduced somatic mutation frequencies and enrichment for the IgG3 isotype (72, 73). Subsequent analyses of replication history and antibody maturation suggested associations between memory B cell phenotype and origin: CD27⁺IgM⁺ and CD27⁻IgG⁺ likely derive from primary germinal center reactions, while CD27⁺IgG⁺ and CD27⁺IgA⁺ cells develop from secondary reactions and CD27⁻IgA⁺ cells mature extrafollicularly (44). In another study, CD27⁺ and CD27⁻ class-switched memory cells showed a genealogical relationship but CD27 expression was not specifically associated with product or precursor cells (55). Together, these studies used evidence of somatic hypermutation, clonal expansion, and/or specificity to previously encountered pathogens to establish the existence of CD27⁻ memory B cells.

The origin and identity of human IgM⁺ memory B cells, a population divided into IgM⁺IgD^{low/-} ("IgM-only") and IgM⁺IgD⁺ subsets, remains a subject of debate. IgM-only cells appear to be germinal center-derived and clonally related to class-switched CD27⁺ memory B cells (74). Conversely, IgM⁺IgD⁺ cells were initially proposed to be either naïve cells with pre-immune antibody mutations, TI response-derived cells, or the circulating counterpart of the

splenic marginal zone B cell population (6, 74, 75). However, a number of studies identified characteristics suggesting a germinal center-dependent origin for significant portions of the IgM⁺IgD⁺ pool, which molecularly, phenotypically, and functionally resemble IgG⁺ memory B cells (6, 45). Taken together, IgM⁺IgD⁺ memory B cells are likely a heterogeneous population with multiple origins and biological niches.

Signals regulating memory B cell heterogeneity

The differentiation fate of activated naïve B cells (and potentially re-activated memory B cells) is impacted directly by pathogen-derived molecular signals and cytokines produced by various immune and non-immune cells in response to the infection (64, 76). Via this process, specific infection cues shape the character of the subsequent antigen-specific memory B cell response and ultimately generate a diverse pool of memory B cells. Murine studies indicate that B cell differentiation can occur in a polarized way, analogous to CD4⁺ T cell polarization, in a process largely dependent upon cytokines: IFN γ drives Th1-like differentiation in B cells, while IL-4 drives Th2-like differentiation patterns (64, 77). These polarized B cells subsequently produce signature cytokines associated with their respective immune response types, undergo specific patterns of antibody isotype class switching, and are imprinted with a trafficking receptor profile (65, 78, 79).

B cells further depend upon toll-like receptors (TLRs), innate sensors of pathogen-associated molecular patterns, to generate appropriate humoral responses. Naïve B cells only express TLRs at low levels, but human memory B cells can express TLRs 1, 2, 6, 7, 9, and 10: TLRs 1, 2, and 6 are plasma membrane-expressed and recognize bacterial antigens, while TLRs 7, and 9 are expressed in endosomes and recognize nucleic acids (TLR10's function is unclear (76)). TLRs are upregulated by cell activation and, by synergizing with BCR and CD40 stimulation, can further promote B cell activation and influence differentiation (80). TLR signaling is critical at multiple stages of B cell development, as GC B cells are particularly sensitive to TLR signaling and plasma cell differentiation directly depends on TLR activation (81-83). Deletion of

TLRs or TLR-associated signaling molecules such as MyD88 in mice have been shown to diminish the magnitude or quality of antibody responses against protein immunogens, viral-like particles, and live replicating pathogens, directly demonstrating the impact of TLR-signaling on developing humoral responses (76). Together, cytokine and TLR stimulation are transduced by a number of signaling pathways that culminate in the activation of transcription factors to subsequently alter gene expression.

Memory B cell migration and tissue distribution

Memory B cells utilize peripheral blood and the lymphatic system to traffic toward lymphoid and non-lymphoid tissues where antigen encounter is most likely. Memory B cells have been identified at mucosal sites, such as the gut and lungs, in the skin, in various lymphoid tissues, and can be readily recruited to sites of inflammation (6, 84-86). The directed migration of lymphocytes into tissues is dependent upon the processes of chemotaxis, the movement of cells toward a chemokine gradient, and cell adhesion. Memory B cell subsets can be targeted toward anatomical sites by expression of specific chemokine receptors and adhesion molecules; for example, gut homing requires expression of the $\alpha 4\beta 7$ integrin and the chemokine receptor CCR9, whereas skin homing involves cutaneous lymphocyte antigen and contributions from chemokine receptors CCR4, CCR6, and CCR10 under different conditions (84, 85).

Lymphocyte entrance into and exit from lymph nodes is perhaps the best described trafficking process (87). B and T cells enter into lymph nodes via afferent lymph or from blood via high endothelial venules (HEVs), and entry is highly dependent upon adhesion molecule interactions: lymphocyte-expressed CD62L interacts with HEV-expressed addressins, causing the cells to slow down, roll along the endothelial surface, engage endothelial ICAM1 and ICAM2 adhesion molecules via lymphocyte function-associated antigen 1, and extravasate between endothelial cells into the lymph node parenchyma (87, 88). Chemokine receptors such as CXCR5 and CCR7 also support lymph node entry and further guide B cell localization within the lymph node: CXCR5 signaling induces B cells and Tfh cells to traffic into B cell follicles, B cell-rich areas

in which TD humoral responses are initiated and matured via the germinal center reaction (21, 22). T cell cortex surrounds these follicles, and CCR7 positions B cells near the T cell border to facilitate B-T cell interactions (19). B cells leaving the lymph node emigrate into the lymphatic system following a sphingosine 1-phosphate (S1P) gradient, from low concentrations in lymph node parenchyma to high blood and lymph concentrations (89). Recent emigrants can travel to and survey additional lymph nodes or enter the blood circulation via the thoracic or right lymphatic ducts (90).

The spleen is a unique lymphoid organ optimized for blood-borne pathogen responses. Spleen is comprised of two main compartments: white pulp, with a T cell zone and B cell follicles that resembles lymph node structure, and red pulp, a macrophage-rich site that filters the blood (91). In humans, follicles are surrounded by a two-layer marginal zone, which contains a specific B cell subset termed marginal zone (MZ) B cells, and a perifollicular zone that further separates the MZ and red pulp (91). Peripheral blood lymphocytes enter the spleen via splenic arterioles that empty into the open circulation of the perifollicular zone, and these cells can subsequently traffic into white pulp in a CCR7 or CXCR5-dependent manner or into the red pulp's splenic sinuses to exit the spleen along an S1P gradient (21, 22, 87, 91). Due to differences in structure and cell composition between rodents and humans (i.e. lack of specific macrophage populations in human splenic marginal zone), the mechanisms of blood sampling and antigen delivery to human MZ B cells are unclear (92); however, MZ B cells are important for TI responses to blood-borne pathogens such as encapsulated bacteria and can quickly differentiate into plasmablasts upon antigen encounter (6). Additionally, MZ B cells function to connect the innate and adaptive immune systems by shuttling between the marginal zone and B cell follicles in an S1P-dependent manner to deliver antigens for initiation of follicular responses (93). Unlike rodents, human MZ B cells are somatically mutated and therefore represent a memory population (92). It is unclear if human MZ B cells can traffic into the periphery or are spleen-restricted (6).

Since cell tracing is difficult in humans, it is often unclear whether B cells identified within human peripheral tissues are transiently surveying for pathogens or reside permanently within

these tissues. The concept of resident memory cells, specific subsets that remain in tissues and do not re-circulate, is well described in the T cell literature but is poorly understood in the context of B cells. To maintain tissue residence, T cells rely on integrins and other receptors to mediate adhesion and actively inhibit egress mechanisms (94). Resident memory T cells (T_{RM}) characteristically express CD69, a C-type lectin that inhibits sphingosine 1-phosphate receptor 1 (S1P1)-regulated egress, and CD103 (αE integrin), which complexes with $\beta 7$ integrin to mediate epithelial cell adhesion (94, 95). While definitive B cell tissue residence markers have not been defined, lymphocyte circulation studies in sheep indicate that specific subsets of B cells do not re-circulate (i.e. are not found in lymph) and likely remain in tissues (96). Plasma cells generally do not re-circulate and are resident in bone marrow and gut lamina propria (34, 35).

1.5 The transcription factor T-bet

T-bet in immunity

The T-box transcription factor family includes a number of genes critical for regulation of developmental processes (97). Named after the *Brachyury*, or *T* gene, family members characteristically contain a highly conserved, 180 amino acid T-box DNA binding domain (98). In 2000, Szabo et al. discovered a novel family member within T cells, which they termed T-bet (T-box expressed in T cells (99)). T-bet was first described as a critical regulator of naïve CD4 T cell differentiation into the T helper 1 (Th1) lineage, but subsequent studies have identified the importance of this transcription factor in regulating development and effector functions of various immune cell types, including CD8 T cells, NK cells, B cells, dendritic cells (DCs), and monocytes (100). T-bet is expressed exclusively within the immune system where it promotes type 1 (Th1) immunity against intracellular pathogens (100).

T-bet is critical for driving both innate and adaptive Th1 responses. Monocytes and DCs are induced to express T-bet by IFN γ stimulation, and DCs require this intrinsic T-bet expression

to appropriately prime Th1 CD4⁺ T cells and shape the subsequent adaptive immune response (101, 102). Innate lymphoid cells, NK cells, invariant NKT cells, and $\gamma\delta$ T cells, separate lineages of innate-like lymphocytes, require T-bet at various developmental checkpoints and to drive effector functions such as IFN γ production (100). Th1 CD4⁺ T cell development and IFN γ production also require intrinsic T-bet expression, and forced expression of T-bet in Th2 and Th17 cells reverts these cells to a quasi-Th1 phenotype (99). CD4⁺ T cells lacking T-bet cannot mount Th1 responses and fail to control intracellular bacterial infections (99, 103). T-bet is also an important regulator of CD8⁺ T cell development and effector functions, although it works cooperatively and often redundantly with the T-box family member Eomesodermin (Eomes (104, 105)). T-bet seems to be particularly important for early production of cytolytic proteins, such as granzyme B, and IFN γ , during CD8⁺ T cell responses, and subsequent Eomes induction can support and complement these effector functions (100). Conversely, memory differentiation is regulated differentially by T-bet and Eomes: T-bet promotes terminal differentiation into the effector phenotype, while Eomes promotes the formation of central memory CD8⁺ T cells (100). Combined T cell receptor stimulation and signaling from inflammatory cytokines such as IFN γ or IL-12 promote T-bet expression in T cells (99, 106).

Mechanistically, T-bet functions both as a transcriptional activator to support Th1 identity and a repressor to prevent the adoption of alternative lineage fates. T-bet actively promotes expression of genes associated with the Th1 phenotype, such as *Ifng* and *Cxcr3*, and T-bet can drive similar gene expression programs between different lineages by binding the same gene targets in each cell type (107). However, T-bet also can promote separate lineage-specific Th1-associated programs in different immune cells (e.g. cytolytic potential in CD8⁺ T cells but not B cells (100)). To support gene expression, T-bet recruits cofactors such as Runx3 for activation of the *Ifng* gene and chromatin remodeling complexes to open target loci (108). For repression, T-bet similarly recruits Bcl-6, a transcriptional repressor, and exploits its repressive function to silence specific non-Th1 genes (109). T-bet can also indirectly repress alternative lineage development by binding to and inactivating GATA3 (Th2 differentiation) and Runx1 (activator of

Rorc, which promotes Th17 differentiation (108)). These diverse gene regulation mechanisms combine to support Th1 cell identity across the immune system.

T-bet in B cells

T-bet expression was first identified in mouse B cells by Glimcher et al., who found that T-bet regulates IFN γ production by B cells following Th1 cytokine (IL-12, IL-18) and CD40 stimulation (99). This B cell-produced IFN γ was suggested to play an important immunoregulatory role by promoting Th1 CD4 T cell development, and, in an analogous way, autocrine Th1-type polarization of B cells (64) (65). However, subsequent studies identified perhaps the most important B cell-specific function of T-bet: regulation of antibody isotype switching to the IgG2a/c isotype (78) (110), the signature antibody isotype of humoral Th1-type immune responses (111). Following completion of isotype switching, IgG2a⁺ memory B cells and plasmablasts continue to depend on T-bet for their survival and functionality (112). Altogether, T-bet appears to function as a Th1 master regulator within the B cell compartment of mice by controlling the early development of IgG2a⁺ B cells from naïve precursors and actively maintaining the integrity of mature IgG2a⁺ memory B cells.

The roles of T-bet in regulating IFN γ production and IgG2a class switching suggest that this transcription factor primes B cells for antiviral responses. Early *in vitro* studies of cytokines or other factors promoting B cell T-bet induction, such as IL-12, IL-18, and anti-CD40 stimulation, have further supported this hypothesis (99). Subsequent studies have additionally identified IFN γ and IL-21 as potent inducers of T-bet expression and IgG2a isotype switching in B cells, particularly when paired with TLR7 or TLR9 stimulation (78, 110, 113-116). As viral nucleic acids can stimulate TLR7 and/or TLR9, and IFN γ and IL-21 are produced by the immune system in response to viral infections, these experiments suggested that viral infection would be ideal for development of the T-bet⁺ B cell subset.

Despite this suggestive body of work, T-bet⁺ B cell involvement in specific antiviral responses was not directly demonstrated until several years later. Using the gamma herpes virus

68 mouse model of viral infection, Rubtsova et al. showed that T-bet⁺ B cells acutely expand, produce anti-ghv68 antibodies, and are necessary to control viremia to low levels (117). More recently, Barnett et al. (2016) used inducible B cell-specific T-bet knockout mice to show that T-bet⁺ B cells are critical for maintaining control of chronic lymphocytic choriomeningitis infection, through both virus-specific IgG2a production and antibody-independent functions (118). By connecting viral loads to the presence of this population, these studies together have established T-bet⁺ B cells as an antiviral subset, with a direct role in controlling multiple murine viral infections.

In humans, T-bet expression has been identified in peripheral blood B cells specifically within the context of inflammatory diseases, including systemic lupus erythematosus (SLE), Crohn's disease, multiple sclerosis, and chronic hepatitis C virus (HCV) infection (119-122). In lymphoid tissue, a subset of B cells termed monocytoïd B cells normally express T-bet, and widespread T-bet expression is detectable in B cells within Th1-type reactive lymphoid tissue lesions and also several neoplastic lesions (123-125). T-bet's induction in human B cells under pathologic, inflammatory conditions raises the question of whether T-bet expression is aberrant or a normal transcriptional regulator of human B cells.

1.6 B cells and HIV infection

HIV and immunopathogenesis

The first description of what would later be recognized as acquired immune deficiency syndrome (AIDS) was made in 1981 with the observations of several homosexual men infected with *Pneumocystis carinii* (126) and developing Kaposi's sarcoma (127). The subsequent identification of a novel retrovirus by multiple groups led to the characterization of the human immunodeficiency virus (HIV (128)). HIV is a single-stranded RNA virus that binds to and enters immune cells using CD4 and a co-receptor (commonly the chemokine receptors CCR5 or CXCR4

(129)). HIV characteristically infects and kills CD4⁺ T cells, leading to the significant loss of CD4⁺ T cell numbers and progressive immunopathology culminating in AIDS and death (130). HIV remains a global health crisis over 30 years after its initial discovery as the causative agent of AIDS, with over 30 million individuals infected and nearly 2 million deaths per year attributed to the infection (131). Attempts to create an HIV vaccine have largely failed due to the virus' extraordinary antigenic diversity driven by high mutation and replication rates and its ability to induce systemic immunopathology.

Following transmission and an eclipse phase when virus is undetectable in the blood, excessive viral replication produces an acute peak in HIV viremia and concomitant loss of CD4⁺ T cells (132). This depletion results in diminished help to B cells and CD8⁺ T cells, negatively impacting the development of both humoral and cellular immunity to HIV (133). CD4⁺ T cell depletion in the GALT is particularly prevalent, and this process contributes to loss of mucosal barrier integrity, microbial translocation, and subsequent systemic inflammation that further impairs HIV immunity (134). Following peak viral load, HIV viremia is subsequently controlled to a set point by CD8⁺ T cell-dependent mechanisms (135). Since cellular and humoral immune responses are unable to achieve viral clearance, most infected individuals maintain a steady state of viremia asymptomatically for years until CD4⁺ T cell numbers further decline and leave individuals susceptible to opportunistic infections (133). HIV disease is characterized by chronic viral replication and immune activation that impairs the functionality of all major immune subsets (133).

The HIV Env antibody response

HIV infection elicits a strong humoral response against a number of viral proteins, with antibodies targeting Env, the HIV surface glycoprotein, mediating the majority of antiviral effects. IgM isotype antibodies emerge first during early acute HIV infection and recognize gp41, the membrane bound Env subunit critical for mediating membrane fusion. These gp41 antibodies subsequently undergo class switching to IgG and IgA but remain largely ineffective (136).

Similarly, antibodies specific for a number of epitopes on gp120, the cap of Env that binds CD4 and co-receptors, begin to appear in a sequential manner approximately 4 weeks after infection but rarely mediate neutralization (136-139). Autologous HIV neutralizing antibodies targeting Env variable regions first appear 4-12 weeks after infection but are unable to neutralize current viral quasispecies (54). A number of studies demonstrate that HIV Env-specific neutralizing antibodies directly drive Env mutation, and some individuals mount early neutralizing responses that significantly pressure the virus (137, 140); however, a situation develops in most chronically infected individuals where the generation of Env sequence diversity greatly outpaces autologous neutralization development, thereby preventing antibody-mediated clearance of HIV (54).

In recent years, technological advances have enabled the isolation and characterization of multiple classes HIV broadly neutralizing antibodies (bNAbs (141)). Unlike typical Env-specific antibodies, bNAbs demonstrate significant neutralizing capacity against diverse heterologous viruses (142). These antibodies develop in a subset of chronically infected individuals only after years of infection, suggesting their maturity requires prolonged viremia and/or immune activation (143). These antibodies function by targeting sites of weakness on the Env protein, including the CD4 binding site, membrane proximal external region, gp120-gp41 interface, and V1-V2 glycan, and are further characterized by abnormal features such as long complementarity determining region 3 (CDR3) regions, excessive somatic hypermutation levels, and increased autoreactivity (143, 144). While bNAbs arise late in the course of infection and are unable to neutralize concurrent dominant HIV variants in the infected individual, their development indicates that the human body is capable of producing antibodies that can neutralize genetically diverse strains of HIV.

B cell hyperactivity and subset alterations

From the earliest descriptions of HIV infection, B cell hyperactivity was evidenced in viremic individuals by lymphadenopathy, hypergammaglobulinemia, and increased activation marker expression, cell turnover, and cell death (145, 146). The B cell compartment is

significantly impacted by HIV infection, demonstrating drastic alterations in cell phenotype, functionality, and the representation of particular subsets (146). Many of these changes are due to the effects of excessive infection-induced cytokines and viral replication products on B cells and other immune cells that regulate B cell development (147). Hyperactivation and many of the B cell subset imbalances can be normalized by lowering immune activation with antiretroviral therapy (ART); however, ART-treated individuals exhibit instances of deficient humoral recall responses and an incomplete restoration of resting memory B cell numbers by ART, suggesting that chronic viremia irreversibly depletes a portion of humoral memory either directly or indirectly through the loss of CD4⁺ T cell help (146, 148). These HIV infection-induced perturbations may impede the development and maintenance of protective B cell and antibody responses, contributing to chronic persistence of the virus in untreated individuals. However, it is important to note that HIV infection does elicit a strong and evolving humoral response that places immunological pressure on the virus (137, 138).

The peripheral B cell compartment in HIV-infected individuals is significantly altered compared to HIV⁻ individuals, with observable differences in the representation of nearly every subset (147). Transitional B cells, recent immature bone marrow emigrants detectable at a low frequency in healthy individuals, are increased in number as a result of CD4⁺ T cell lymphocytopenia and increased IL-7 levels (149). The inflammatory environment of HIV infection is thought to drive polyclonal B cell activation and terminal differentiation and to promote memory B cell apoptosis; as such, decreases in naïve and resting memory B cells and increased plasmablast numbers are commonly observed (147). Viremic HIV infection is also associated with the expansion of atypical memory B cells, subsets present at low to undetectable levels in healthy individuals that can be identified by a loss of CD21 expression. These cells are commonly subsetted and defined as activated (CD21⁻CD27⁺) and tissue-like memory (CD21⁻CD27⁻) B cells, and the latter has been described as “exhausted” due to its low proliferative capacity following BCR (150, 151).

The B cell response to HIV

Many aspects of the HIV-specific B cell response are poorly understood, due to the historical lack of cell identification technologies and the field's general emphasis on studying antibodies (147). Recently, HIV Env probes were developed to enable flow cytometric sorting of virus-specific memory B cells for cloning of HIV-specific antibodies (152, 153). This process is commonly used for the identification and characterization of bNAbs from infected individuals, but these probes also afford the opportunity to assess characteristics of the HIV-specific memory B cell compartment itself. Studies of HIV-specific B cells during acute infection are lacking since individuals are rarely diagnosed before the chronic phase of infection, but HIV Env probe-based work suggests much of the early gp41-focused IgM isotype antibody response is generated from cross-reactive, commensal bacteria-specific B cells (154). Interestingly, analyses of chronically infected individuals found that HIV-specific responses are concentrated in activated and tissue-like memory B cells instead of classical memory B cells, a phenomenon often interpreted to reflect immune dysfunction (155). However, the detrimental impact of an atypical memory cell-biased (versus classical memory-biased) response has not been directly demonstrated, and the mechanisms shifting HIV-specific B cell development into the atypical memory path are unclear.

1.7 Thesis goals

Complex viruses such as influenza and HIV have moved the vaccinology field from its empirical roots into a new era of rational-based vaccine design requiring careful characterization of protective immune correlates. To this end, identification of the mechanisms regulating human antiviral B cell responses will provide biomarkers to assess the immunogenicity of vaccine candidates and molecular targets for intervention to improve vaccine-induced immune responses. Despite significant advances in antibody characterization, factors regulating antiviral humoral immunity and the specific B cells mediating acute responses remain undefined. As such, the goal

of this thesis was to better define the cells and mechanisms that promote humoral antiviral responses by characterizing T-bet's role in the human B cell compartment and assessing T-bet⁺ B cells' involvement in the HIV immune response

CHAPTER 2

IDENTIFICATION AND CHARACTERIZATION OF HUMAN T-BET-EXPRESSING B LYMPHOCYTES

2.1 Summary

Humoral immunity is critical for viral control, but the identity and mechanisms regulating human antiviral B cells are unclear. Here, we characterized human B cells expressing the transcription factor T-bet and analyzed their dynamics during live viral vaccinations. T-bet⁺ B cells, a relatively small component of the peripheral B cell compartment in healthy individuals, demonstrated a memory phenotype and were enriched for the antiviral immunoglobulin isotypes IgG1 and IgG3. We identified T-bet^{high} and T-bet^{low} B cell subsets with divergent phenotypic profiles and responses to vaccination: T-bet^{high} cells were highly activated, transcriptionally distinct from resting memory B cells and plasmablasts, and expanded acutely and transiently following yellow fever and vaccinia virus vaccinations. Conversely, T-bet^{low} cells displayed a resting phenotype and were unresponsive to acute viral infection in most donors. T-bet^{low} cells were readily identified in peripheral blood, lymphoid tissues, and in thoracic duct fluid, while T-bet^{high} cells were found primarily in the peripheral blood and in low numbers within the spleen. Together, these findings suggest T-bet regulates antiviral immunoglobulin isotype switching and development of a distinct T-bet^{high} B cell subset that is responsive to viral replication and/or inflammatory signals in healthy individuals.

2.2 Introduction

The humoral immune system is critical for control of multiple viruses during both acute and chronic phases of infection (118, 156), and most effective vaccines are thought to function by eliciting a protective humoral response (4). Humoral immunity is coordinated by memory B cells, antigen-specific subsets that can regulate the developing immune response via functions such as antigen presentation, cytokine production, or differentiation into antibody-secreting cells (62, 157, 158). Memory B cells can also express different antibody isotypes which fulfill diverse spatiotemporal and pathogen-specific roles upon secretion (6, 52). Heterogeneity has been demonstrated within the origins, development, and functional capacity of human memory B cell populations differentiated by a variety of cell surface markers (6). Recent studies have begun to assess the contributions of divergent B cell subsets during active immune responses using antigen-specific probes (155, 159), but the identity and regulation of memory B cells responding to active human viral infections remain poorly understood.

Recently, several studies have identified atypical memory B cells as bona fide antigen-experienced subsets (44, 55, 72, 73, 151). These cells differ from classical memory B cells by their activated phenotype (151) and/or absence of CD27 expression (44, 55, 72, 73), a traditional marker of B cell memory (68, 69), but show evidence of a germinal center origin based upon antibody isotype switching and somatic hypermutation (44, 55, 72, 73, 151). Atypical memory B cells have subsequently been described in the context of chronic inflammatory settings in which they are abnormally expanded (73, 121, 151, 160-163), and their refractory response to normal B cell receptor stimulation has led to the hypothesis that they are an anergic byproduct of excessive inflammation (151, 162). However, the immunological role for these populations is unclear.

Transcription factors are critical regulators of memory B cell identity and function that can translate pathogen-specific cues into induction of appropriate humoral responses (76, 112, 113, 164). Recent studies identified the immune cell-specific transcription factor T-bet as a critical regulator of murine antiviral B cell responses (117, 118). T-bet was originally described to control

CD4⁺ Th1 cell development and functionality (99), but T-bet also plays a role in B cell differentiation (64, 78). In mice, T-bet expression is required for isotype switching, functionality, and survival of IgG2a/c⁺ memory B cells (78, 110, 112) and can also regulate the expression of the antiviral cytokine IFN γ and the inflammatory homing receptor CXCR3 in this population (65, 79). Several groups recently examined the direct role of T-bet⁺ B cells during murine viral infections: gamma herpes virus 68 induces an expansion of T-bet⁺ B cells, the absence of which leads to infection exacerbation (117). Similarly, chronic lymphochoriomeningitis (LCMV) viremia is controlled to low levels only in the presence of T-bet⁺ B cells via a chiefly IgG2a-dependent mechanism (118). B cells expressing either *TBX21* transcript or T-bet protein have been described in the context of autoimmune diseases, chronic hepatitis C infection, and malaria infection (120, 121, 162, 165, 166), but the biological niche of this population in healthy humans has not been defined.

In this study, we characterize the phenotypic and transcriptional profiles of human T-bet⁺ B cells and examine their dynamics during acute human viral infections. We identified T-bet⁺ B cells in healthy human peripheral blood as an antigen-experienced population that demonstrates a potentially antiviral phenotype. Unlike T-bet⁻ memory B cells, a subset of T-bet⁺ B cells is activated and transiently expands in response to acute viral infections. Our data identify T-bet induction in the B cell compartment by human viruses and suggest that T-bet⁺ B cells participate in the antiviral response.

2.3 Results

2.3.1 Human peripheral blood B cells can express the Th1 transcription factor T-bet

Human T cells and NK cells express the Th1 transcription factors T-bet and Eomesodermin (Eomes), which regulate cell differentiation and effector functions (100, 167-170). We set out to identify B cells expressing these transcription factors in healthy individuals and to compare expression levels to other peripheral blood mononuclear cells (Figure 1A). While a relatively low frequency of B cells (~10%) expressed T-bet compared to CD4⁺ T cells (~30%), CD8⁺ T cells (~65%), and NK cells (~100%), T-bet was consistently detected in B cells of all tested donors (Figures 1B and 1C). T-bet expression levels (median fluorescence intensity, or MFI) within the T-bet⁺ B cell subset were comparable to T-bet⁺ T cells, while NK cells demonstrated the greatest T-bet MFI (Figures 1B and 1D). Unlike T and NK cells, Eomes expression was not detected in B cells (Figures 1E and 1F). Monocytes and CD11c⁺ dendritic cells did not express either T-bet or Eomes (Figures 1B, 1C, 1E, 1F, and data not shown), suggesting Th1 transcription factors do not regulate human myeloid cells at rest.

T-bet expression in T cells is restricted specifically to memory subsets (100). In order to define T-bet⁺ B cell populations in human blood, we developed a panel to assess all major peripheral B cell subsets through expression of CD10, CD38, CD27, CD21, and immunoglobulin (Ig) D (Figure 2A). We found that significantly more memory B cells expressed T-bet compared to transitional B cells, naïve B cells, and plasmablasts (Figures 2B and 2C). As T-bet is known to regulate antibody class switching to IgG2a/c in mice (78, 110), we further assessed the relationship between T-bet and human memory B cell BCR isotypes (Figure 2D). T-bet was expressed at the highest frequency by IgG⁺ cells, specifically those expressing IgG1 or IgG3, which are critical for antiviral responses ((52); Figure 2E). However, T-bet expression could also be detected to a lesser degree in subsets of B cells expressing the other tested antibody isotypes (Figure 2E).

Figure 1

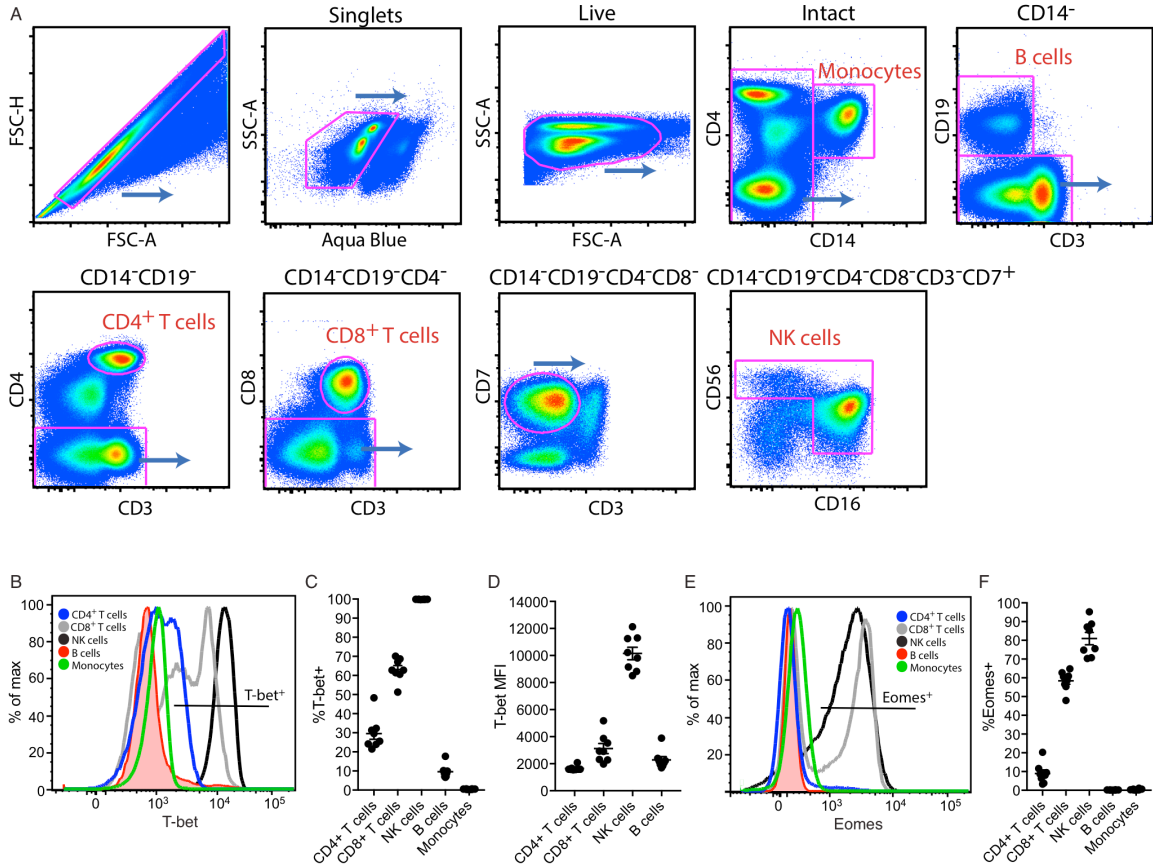


Figure 1: T-bet expression in peripheral blood lymphocytes and monocytes

(A) Gating scheme depicting the identification of major blood immune subsets in a healthy individual; monocytes, CD3⁻CD14⁺CD19⁻; B cells, CD3⁻CD14⁻CD19⁺; CD4⁺ T cells, CD3⁺CD14⁻CD19⁻CD4⁺CD8⁻; CD8⁺ T cells, CD3⁺CD14⁻CD19⁻CD4⁻CD8⁺; NK cells, CD3⁻CD14⁻CD19⁻CD4⁻CD8⁻CD7⁺CD56⁺/CD16⁺. (B) Histogram depicting T-bet expression per subset from a representative individual; CD4⁺ T cells, blue; CD8⁺ T cells, grey; NK cells, black; B cells, red; monocytes, green. (C) Frequency of T-bet expression of each subset in an 8-donor cohort. (D) T-bet MFI of T-bet⁺ cells per subset. (E) Histogram depicting Eomes expression of each subset from a representative individual. (F) Frequency of Eomes expression per subset.

Figure 2

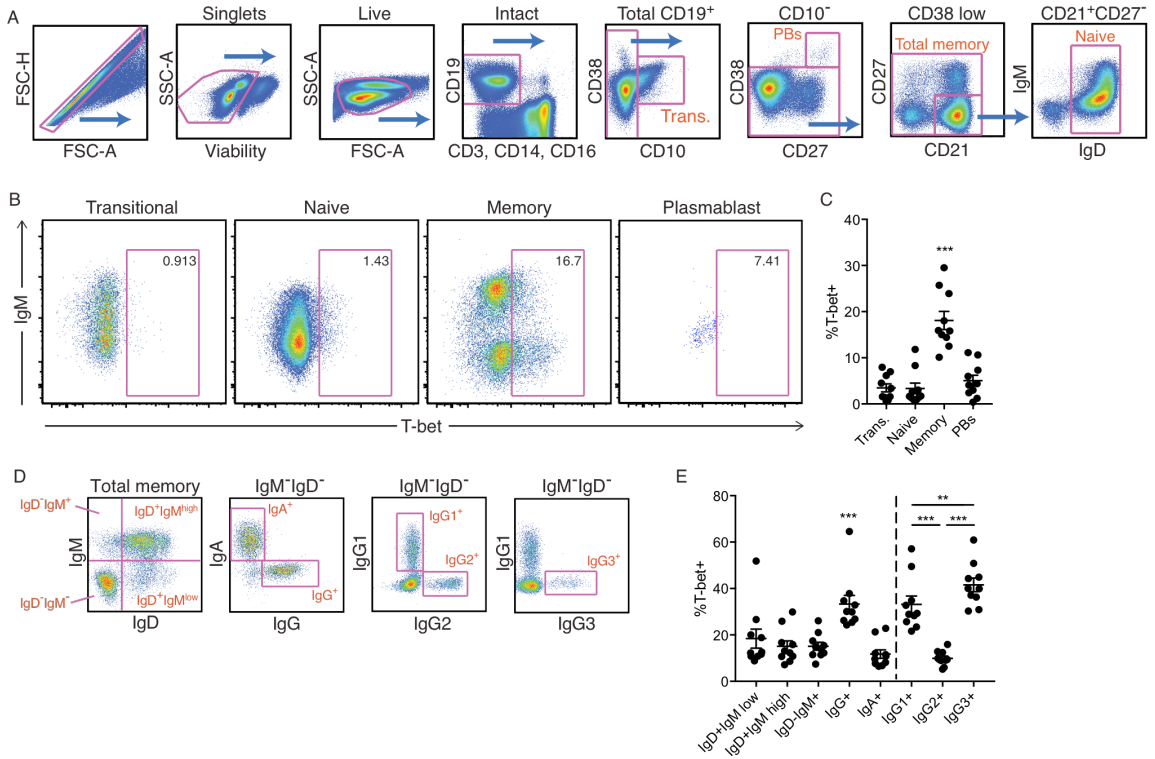


Figure 2: T-bet expression in B cell subsets and by antibody isotype

(A) Representative gating scheme for the identification of B cell subsets named in red from the peripheral blood of a healthy human donor. Trans., transitional, CD19⁺CD10⁺CD38^{mid}CD27⁻; PBs, plasmablasts, CD19⁺CD10⁺CD38^{high}CD27^{high}; naïve, CD19⁺CD10⁺CD38^{low}CD21⁺CD27⁻IgD⁺; total memory cells, CD19⁺CD10⁺CD38^{low} excluding the naïve (CD21⁺CD27⁻) population. **(B)** Representative flow cytometry plots of T-bet expression in peripheral blood B cell subsets from a single donor. **(C)** T-bet expression frequency per subset in a 10-donor cohort. Bars on these and all following plots represent mean \pm standard error. **(D)** Representative identification of memory B cell surface-expressed antibody (BCR) isotypes named in red. **(E)** T-bet expression frequency of memory B cells expressing different antibody isotypes. Statistical comparisons in C and E calculated using repeated measures one-way ANOVA with Tukey's multiple comparisons test. ** denotes a P value $0.01 > P \geq 0.001$. *** denotes a P value $P < 0.001$.

2.3.2 Identification of T-bet^{high} and T-bet^{low} B cell subsets

To further define the characteristics of T-bet⁺ memory B cells, we used CD21 and CD27 to delineate several previously described populations ((151); Figure 3A). The two CD21⁻ subsets, activated memory (AM, CD21⁻CD27⁺) and tissue-like memory (TLM, CD21⁻CD27⁻) B cells, expressed T-bet at the highest frequencies (Figure 3B) and at significantly higher levels per cell (Figure 3C) compared to resting memory (RM, CD21⁺CD27⁺) cells, indicating a prominent role for T-bet in regulation of the peripheral CD21⁻ memory B cell compartment. Interestingly, we identified a bimodal expression pattern of T-bet in AM and TLM subsets (Figure 3D); we therefore explored additional cell surface receptors to better demarcate CD21⁻T-bet⁺ B cells and found that this subset also highly expresses the inhibitory receptor CD85j (Figures 3E-G). The CD21⁻T-bet^{high}CD85j^{high} population (hereafter referred to as T-bet^{high}CD85j^{high} cells) was comprised of approximately half the TLM population and nearly all of the AM population, whereas the remaining TLM cells represent the CD21⁻CD85j^{low} population (Figures 3H and 3I). In summary, we identified two populations of T-bet⁺ B cells: a RM subset expressing T-bet at low levels (T-bet^{low} cells) and a subset distinguished by a lack of CD21 expression and high levels of CD85j that represents the main T-bet^{high} B cell population in the peripheral blood of healthy human donors.

2.3.3 T-bet^{high}CD85j^{high} B cells represent a transcriptionally and phenotypically distinct memory subset

Seeking to better define this novel subset, we next investigated the transcriptional environment of T-bet^{high} B cells, using the CD85j^{high} phenotype as a surrogate marker for Fluidigm Biomark quantitative RNA analysis. Figure 4A depicts transcript expression levels of 91 selected target genes, including transcription factors, activation and inhibitory receptors, trafficking receptors, cell survival/death proteins, and other B cell-relevant genes within sorted B cell subsets from four

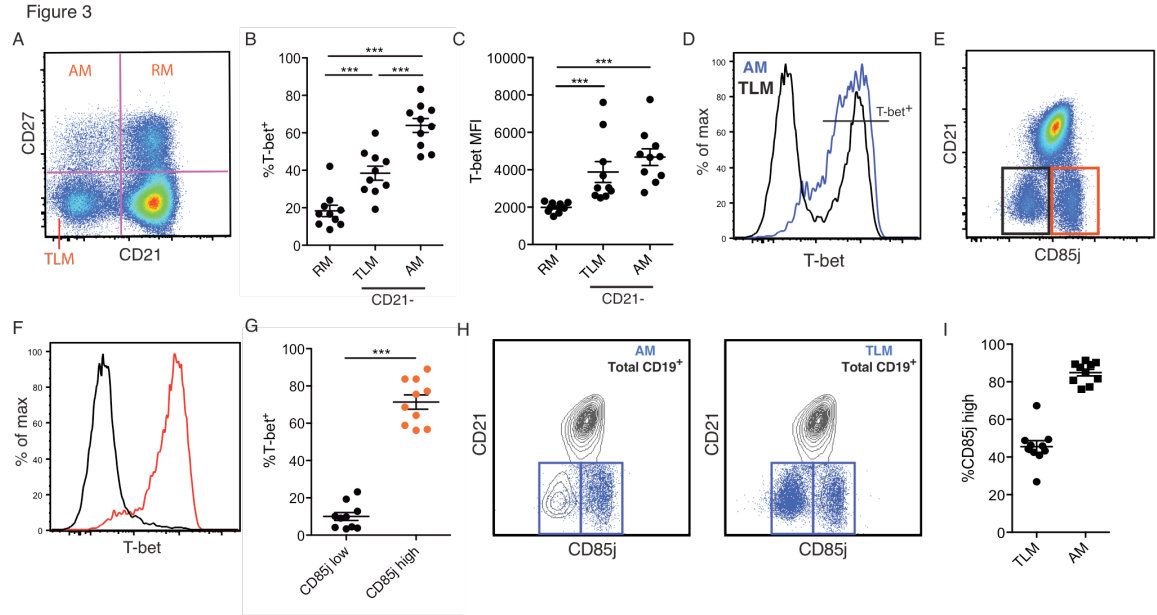


Figure 3: T-bet expression in peripheral blood memory B cell subsets

(A) CD21/CD27-based gating scheme of total CD38^{low}CD10⁻ B cells from a representative donor; activating memory, AM, CD27⁺CD21⁻; resting memory, RM, CD27⁺CD21⁺; tissue-like memory, TLM, CD27⁻CD21⁻. (B) T-bet expression frequency of CD21/CD27-derived B cell subsets. (C) T-bet median fluorescence intensity (MFI) of T-bet⁺ cells from each B cell subset. (D) AM (blue) and TLM (black) T-bet expression histogram from a representative donor. (E) Gating of CD21⁻CD85j^{high} and CD21⁻CD85j^{low} B cell subsets from a representative donor's total CD38^{low}CD10⁻ B cells. (F) T-bet expression histogram of CD85j-gated B cell subsets from donor depicted in E. (G) T-bet expression frequency of CD85j-gated B cell subsets in 10-donor cohort. (H) Flow cytometry plots depicting the gating of AM and TLM into CD85j^{high} and CD85j^{low} populations. Total B cells are depicted in black contour plot; TLM/AM subsets are depicted in blue dot plot. (I) Frequency of AM and TLM subsets demonstrating the CD85j high phenotype (n=10 donors). Statistical comparisons in B and C calculated using repeated measures one-way ANOVA with Tukey's multiple comparisons test. Statistical comparison in G calculated using paired t-test. *** denotes a P value < 0.001.

healthy donors. Unbiased clustering analysis of gene expression data demonstrated consistent clustering of B cell subsets between donors, indicating our chosen panel clearly differentiates peripheral B cell populations (Figure 4A). T-bet^{high}CD85j^{high} cells' transcriptional signature included highest expression of the antibody class switching and somatic hypermutation enzyme *AICDA*, the glycosylation enzyme *B4GALT3*, transcription factors *IKZF1* (Ikaro) and *SOX5*, the inhibitory receptor *SIGLEC6*, and, importantly, *TBX21* (T-bet) (Figure 4A). CD85j high cells shared high expression of *CCR6*, *FCRL3*, *FCRL4*, *NFIL3*, *PBX4*, and *TNF* with RM cells, *FCRL5* and *TOX2* with plasmablasts, and *BATF*, *CASP3*, *CD27*, *FAS*, *FUCA2*, *FUT8*, and *POU2AF1* with both populations (Figure 4A).

To interrogate transcriptional similarity between T-bet^{high}CD85j^{high} cells and the other sorted B cell subsets in an unbiased way, we performed t-distributed stochastic neighbor embedding (tSNE) analysis of the transcript expression results (Figure 4B). T-bet^{high}CD85j^{high} cells were transcriptionally dissimilar to antigen-inexperienced B cells and clustered near to, but distinct from, resting memory B cells and plasmablasts, further suggesting this is an antigen-experienced population. Interestingly, CD85j low B cells, the CD21⁺T-bet⁻ counterpart of our cells of interest, were more similar to the antigen-inexperienced subsets (naïve, transitional; Figure 4B), suggesting these are relatively unrelated to T-bet^{high}CD85j^{high} cells and confirming the previously described population heterogeneity within the TLM phenotype (CD21⁺CD27⁻; (171)).

We further assessed phenotypes of T-bet^{high}CD85j^{high} cells at the protein level and found this subset also highly expressed several homing receptors, activation markers, additional inhibitory receptors (Figure 4C), and demonstrated a diverse repertoire of antibody isotypes (Figure 4D). Interestingly, T-bet^{high}CD85j^{high} cells highly expressed the transcription factor irrespective of surface Ig isotype expression (Figure 4E), suggesting additional Ig isotype-independent roles for T-bet in this population. Taken together, comparative transcriptional and phenotypic studies identify T-bet^{high}CD85j^{high} cells as a highly activated, antigen-experienced population characterized by distinctive homing receptor and transcriptional profiles.

Figure 4
A

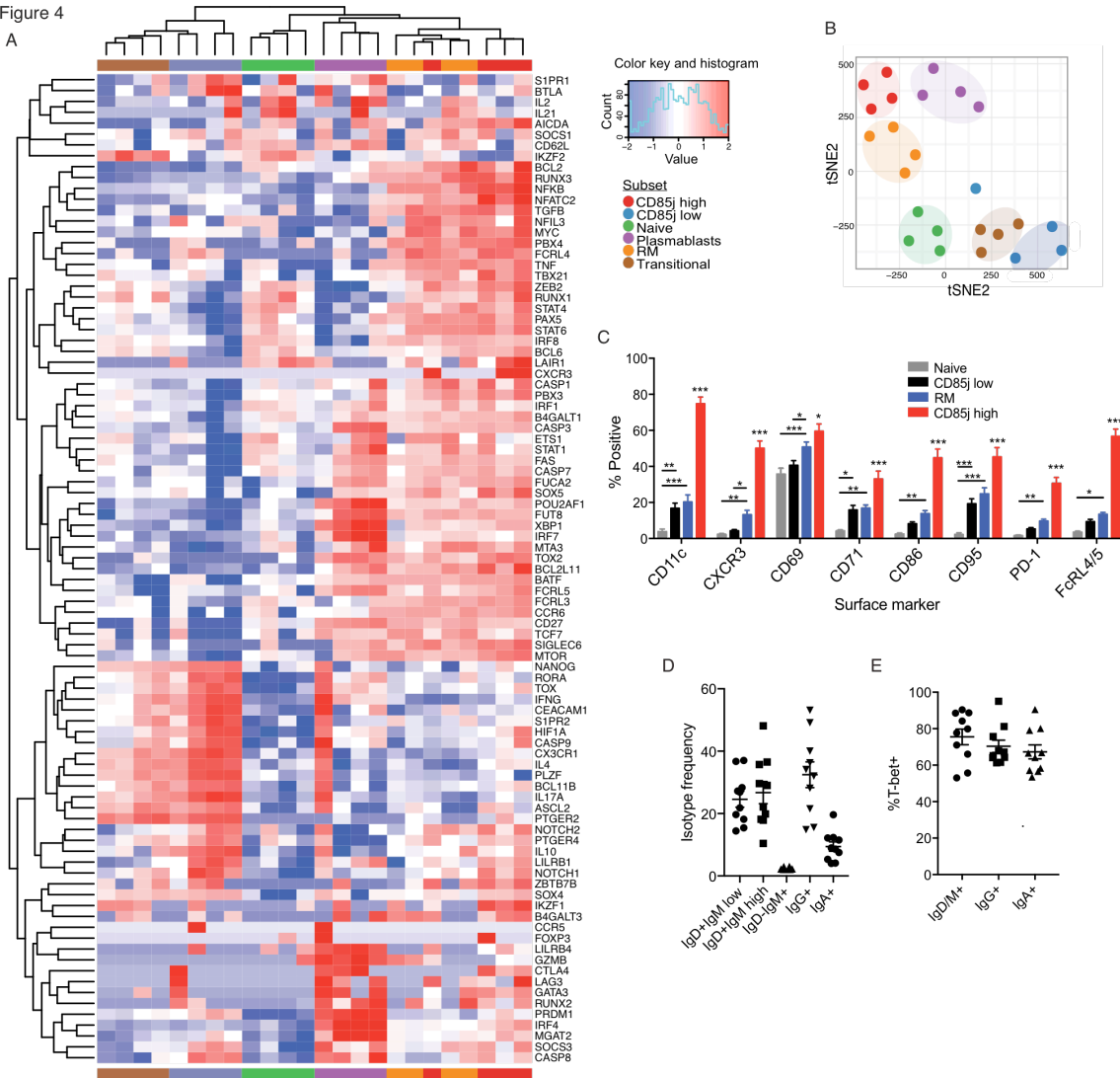


Figure 4: Transcriptional and phenotypic analyses of T-bet^{high}CD85^j^{high} cells and other B cell subsets

(A) Heat map depicting relative RNA transcript expression levels for 91 targets (one per row) in HIV-negative donors (n=4). Each column represents a specific B cell subset (colored bars above and below heat map) sorted from one donor. (B) tSNE analysis of transcriptional relationships between sorted B cell subsets from four healthy donors. Each color represents a sorted B cell subset and clusters are highlighted with the corresponding color. (C) Expression frequency of homing receptors (CD11c, CXCR3), activation markers (CD69, CD71, CD86, CD95), and inhibitory receptors (PD-1, FcRL4/5) by CD38^{low}CD10⁻ B cell subsets. (D) Frequency of antibody isotypes expressed by T-bet^{high}CD85^j^{high} cells (n=10). (E) T-bet expression frequency of T-bet^{high}CD85^j^{high} cells separated by Ig isotype (n=10). Statistical comparisons in C and E

calculated using repeated measures one-way ANOVA with Tukey's multiple comparisons test. No statistical differences were observed between isotypes in E. Statistical comparison in I calculated using paired t-test. * denotes a P value $0.05 > P \geq 0.01$. ** denotes a P value $0.01 > P \geq 0.001$. *** denotes a P value < 0.001 .

2.3.4 Divergent circulatory patterns between T-bet^{high} and T-bet^{low} B cells

We hypothesized that the differing phenotypes of T-bet^{high} and T-bet^{low} B cells would impact their migratory capacity. To understand the circulatory potential of these subsets, we compared the composition of B cell compartments in peripheral blood (PBMC) to the thoracic duct (TD), the body's largest lymphatic vessel that collects lymph draining from both lymphoid and non-lymphoid tissues and empties into the blood (90). We isolated mononuclear cells from paired peripheral blood and thoracic duct fluid (chyle) samples obtained from adults undergoing interventional surgery (see Methods). Compared to PBMC, TD generally lacked myeloid cells and was comprised mainly of T cells, B cells, and a small number of NK cells (data not shown). We assessed the main B cell subset phenotypes found in healthy human blood (Figure 2A) and identified the presence of transitional, naïve, memory, and plasmablast populations in TD of all donors (Figure 5A). Transitional B cell and plasmablast frequencies were similar between PBMC and TD compartments (data not shown), while memory B cells were underrepresented in TD compared to PBMC and naïve cells were more frequent in TD (Figure 5B). Overall, the B cell compartment within these two anatomical sites was similar.

We next investigated T-bet expression by TD B cells and consistently identified diminished frequencies of total T-bet⁺ memory B cells in TD compared to PBMC (Figures 5C and 10D), suggesting some T-bet⁺ B cell subsets may not migrate through the thoracic duct. To understand this phenomenon, we examined T-bet^{high} cells (CD21⁻T-bet^{high}CD85j^{high} phenotype; see Figures 3E and 3F) and found that this subset was virtually absent from the TD B cell compartment, despite detectable T-bet^{high} cell frequencies in PBMC of all matched donors (Figures 5E and 5F). Conversely, T-bet^{low} cells, which could be readily identified within the CD21⁺ population by CXCR3 expression (Figures 5G and 5H), were present at comparable frequencies in both PBMC and TD (Figure 5I). These findings suggest differential circulatory capacities of T-bet^{high} and T-bet^{low} cells: T-bet^{low}CXCR3⁺ cells migrate through the thoracic duct and are able to re-circulate back into blood, while T-bet^{high} cells rarely enter into lymph.

Figure 5

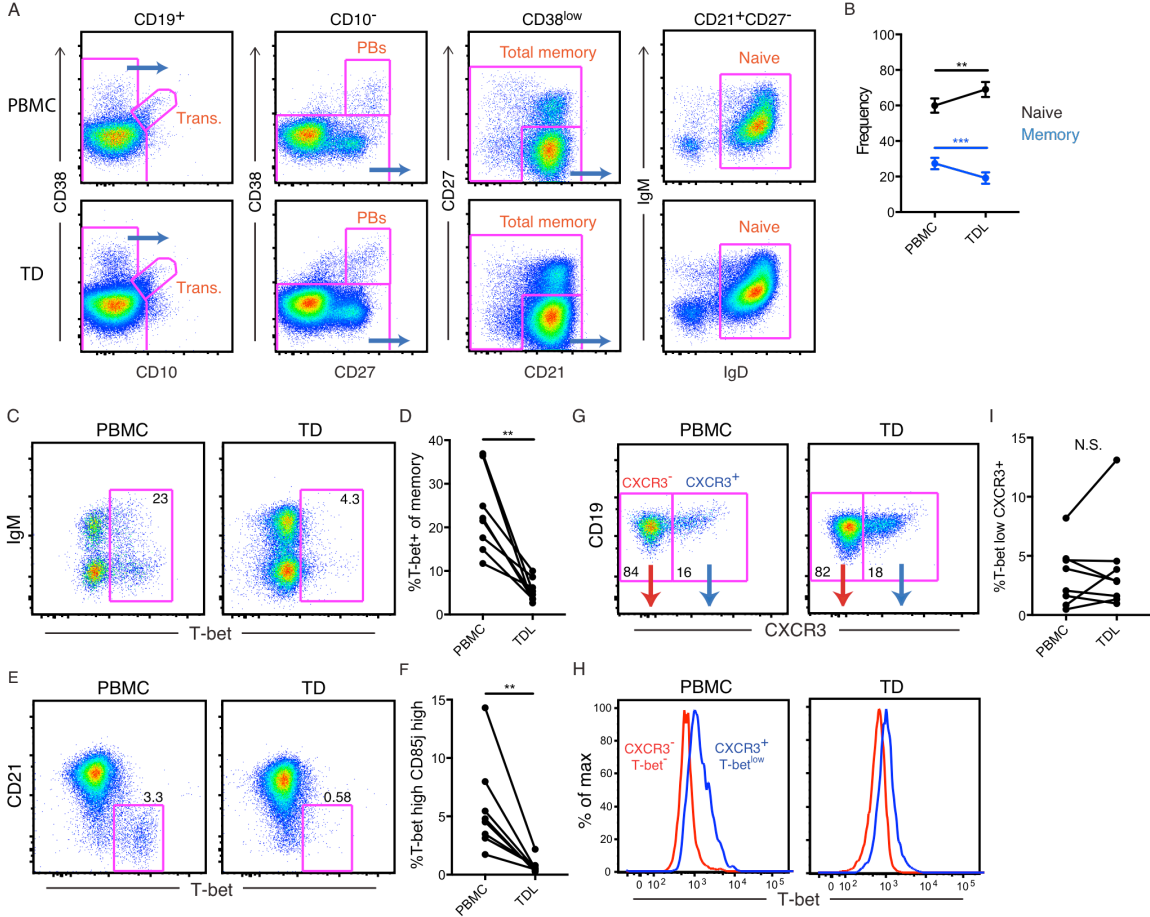


Figure 5: B cell subset distribution and T-bet expression in paired peripheral blood and thoracic duct samples

(A) Gating scheme depicting identification of major B cell subsets as defined in Figure 2A from a representative donor's peripheral blood (PBMC) and thoracic duct fluid (TD). (B) Frequency of naïve (black) and total memory (blue) B cells in 8-donor cohort. (C) Representative flow cytometry plots of T-bet expression in total memory B cells from a representative donor's PBMC and TD. (D) T-bet expression frequency in total memory B cells from PBMC and TD in 8-donor cohort. (E) Identification of T-bet^{high}CD85j^{high} subset within total B cells from a representative donor's PBMC and TD. (F) Frequency of T-bet^{high}CD85j^{high} subset within total B cells in 8-donor cohort. (G) Identification of CXCR3⁺ (blue) and CXCR3⁻ (red) subsets within RM (CD21⁺CD27⁺) population of PBMC and TD. (H) T-bet expression histograms of CXCR3⁺ (blue) and CXCR3⁻ (red) subsets from G in PBMC and TD. (I) Frequency of T-bet^{low}CXCR3⁺ subset within total B cells in 8-donor cohort. Statistical comparisons in B, D, F, and I calculated using paired t-test. ** denotes a P value 0.01 > P ≥ 0.001.

2.3.5 Human spleen harbors a large T-bet-expressing B cell population

Having identified diverse circulatory patterns, we next investigated the tissue distribution of T-bet⁺ B cell subsets. We assessed T-bet expression in B cells obtained from different lymphoid tissues, including iliac lymph nodes (iLN), mesenteric lymph nodes (mLN), tonsils, and spleens that were acquired following surgery (Figure 6A). Iliac lymph node, mesenteric lymph node, and tonsil B cells expressed T-bet at a similar, albeit reduced, frequency compared to PBMC B cells (Figures 6A and 6B); however, MFI of the T-bet⁺ population in each case was significantly lower than that of PBMC B cells (Figure 6C). To determine the types of lymphoid tissue B cells expressing T-bet, we examined major lymph node B cell subsets and identified six donor samples for analysis (two mLN, four tonsil) in which each of the lymphoid populations was sufficiently represented (Figure 6D). T-bet was not characteristically expressed by any specific lymphoid tissue B cell subset, and low T-bet MFI made detection difficult (Figure 6E); however, germinal center B cells (GC; CD38^{mid}Bcl-6⁺CD10⁺Ki67⁺CD21⁺) and CD27⁺ memory B cells exhibited increased T-bet MFI compared to naïve and germinal center-like (GC-like; CD38^{mid}Bcl-6⁻CD10⁺Ki67⁺CD21⁻) B cells (Figures 6E and 6F). Notably, T-bet expression in lymphoid tissues did not associate with diminished CD21 expression as it does in PBMC (Figure 3). In summary, T-bet is expressed only at low levels (frequency and MFI) in iLN, mLN, and tonsil in the absence of an apparent, active immune response, specifically in the GC and CD27⁺ memory subsets.

The spleen is a physiologically distinct lymphoid organ comprised of a white pulp region bearing similarities to lymph nodes and a red pulp region in which macrophages directly sample the blood for antigens (91). The spleen's B cell compartment also differed significantly from the other assessed lymphoid organs, as spleen samples contained a large memory B cell population but few GC or GC-like B cells (data not shown). Interestingly, splenic B cells expressed T-bet at a significantly higher frequency and MFI than PBMC, iLN, mLN, and tonsil B cells (Figures 6A, 6B, and 6C), suggesting the spleen may characteristically harbor a large T-bet⁺ B cell population. These T-bet⁺ B cells were comprised of two distinct subsets: a small CD21⁻T-bet^{high}CD85j^{high} B

Figure 6

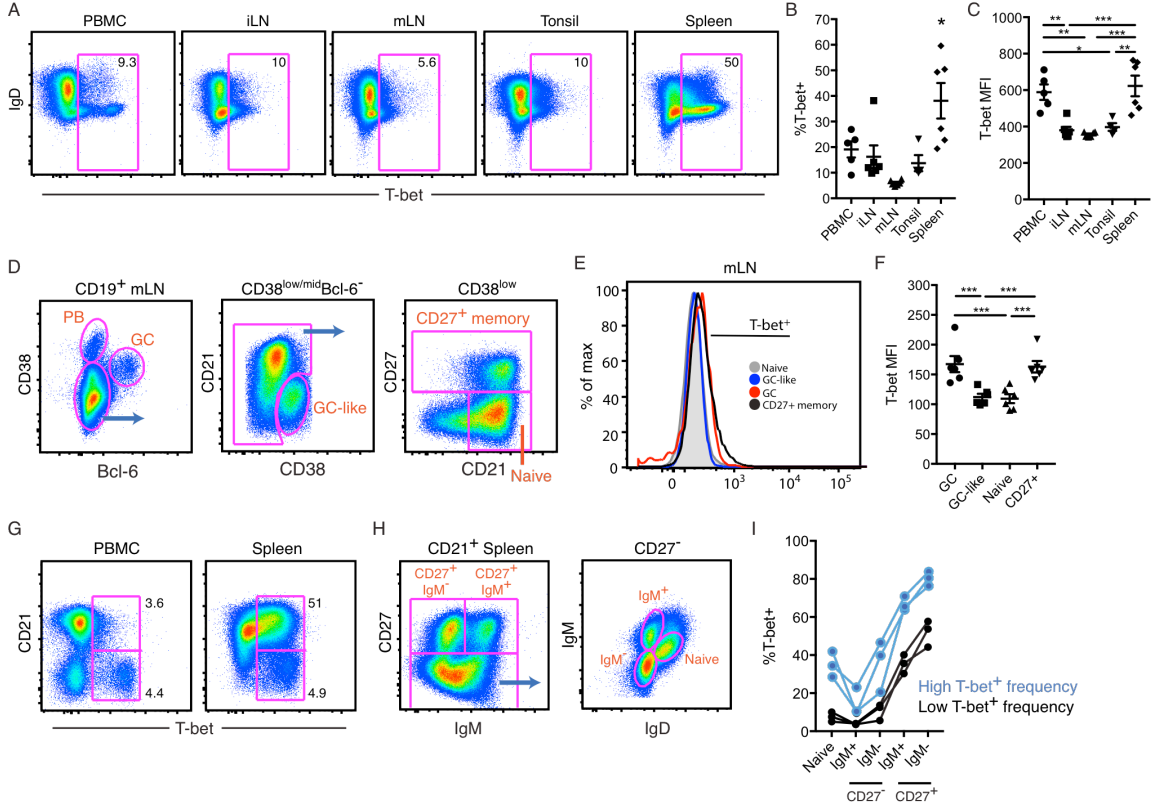


Figure 6: T-bet expression in iliac lymph nodes, mesenteric lymph nodes, tonsil, and spleen

(A) Flow plots depicting representative T-bet expression in total B cells from various unpaired lymphoid tissues; peripheral blood, PBMC; iliac lymph node, iLN; mesenteric lymph node, mLN. (B) Frequency of T-bet expression per lymphoid tissue B cell population; 6 PBMC, 6 iLN, 6 mLN, 4 tonsil, 6 spleen samples. (C) T-bet MFI of the T-bet⁺ B cells from each lymphoid tissue. (D) Identification of lymph node subsets in a representative mLN, with subsets named in red; germinal center B cells, GC, CD38^{mid}Bcl-6⁺CD10⁺Ki67⁺CD21⁺; germinal center-like B cells, GC-like, CD38^{mid}Bcl-6⁻CD10⁺Ki67⁺CD21⁻; plasmablasts, PB, CD38^{high}; CD27⁺ memory, CD38^{low}CD27⁺; naïve, CD38^{low}CD27⁻CD21⁺. (E) T-bet histogram of lymph node subsets from a representative mLN; naïve, grey; GC-like, blue; GC, red; CD27⁺ memory, black. (F) T-bet MFI of lymph node subsets in 6-donor cohort (2 mLN, 4 tonsil). (G) Identification of CD21⁺T-bet⁺ and CD21⁻T-bet⁺ subsets in PBMC and spleen of two representative donors. (H) Identification of CD21⁺ splenic subsets named in red from a representative donor. (I) T-bet expression frequency of splenic CD21⁺ subsets in 6 donor cohort split into two groups: donors with high (>40%; blue) and low (<40%; black) total frequencies of T-bet expression in the spleen in (see Figure 6B). Statistical comparisons in B, C, and F calculated using one-way ANOVA with Tukey's multiple

comparisons test. * denotes a P value $0.05 > P \geq 0.01$; ** denotes a P value $0.01 > P \geq 0.001$. *** denotes a P value < 0.001 .

cell population that phenotypically resembled its PBMC counterpart (Figure 6G), and a large CD21⁺T-bet⁺ B cell population that dwarfed the PBMC CD21⁺T-bet⁺ subset in size and accounted for the majority of T-bet⁺ B cells in spleen (Figure 6G).

To better define splenic CD21⁺T-bet⁺ B cells, we investigated the association between T-bet and diverse phenotypes within the CD21⁺ population (Figure 6H). T-bet was detected in all CD21⁺ memory subsets examined, including isotype switched (IgM⁻), unswitched (IgM⁺), CD27⁺, and CD27⁻ populations, with CD27⁺IgM⁻ memory B cells expressing T-bet at the highest frequency (Figure 6I). We also observed T-bet expression by phenotypically naïve B cells (CD21⁺CD27⁻IgD⁺), suggesting T-bet induction may precede the development of a memory B cell phenotype in this tissue (Figure 6I). Interestingly, individuals with particularly high T-bet⁺ splenic B cell frequencies (>40% T-bet⁺; Figure 6B) did not exhibit expansion of a particular T-bet-expressing subset; instead, they maintained a phenotypically diverse splenic CD21⁺T-bet⁺ B cell pool with elevated T-bet expression frequencies across all subsets (Figure 6I). Together, these findings suggest human spleen represents a reservoir for members of the T-bet⁺ B cell population.

2.3.6 T-bet⁺ B cells are induced during acute viral infections

T-bet⁺ B cells have previously been shown to expand during acute LCMV and ghv68 infections in mice (117, 118); we therefore asked whether human T-bet⁺ B cells might similarly be induced during human acute viral infections. To assess this, we examined B cell responses in yellow fever (YFV) and vaccinia (VV) virus vaccinated human subjects. These replicating live virus vaccines are thought to elicit durable protection via antibody-mediated mechanisms (4, 172-174), suggesting a critical role for B cells in this process. We analyzed T-bet expression within total memory B cells collected from the peripheral blood of vaccinated individuals on the day of vaccination, at weekly acute time points, and at a final time point ranging from 2 to 8 months post-vaccination (Table 1). We identified the emergence of a T-bet⁺ population as early as two weeks

Vaccinia	Donor ID	Week 0	Week 1	Week 2	Week 3	Week 4	Week 25+
	12	Pre	7	14	21	28	180
	13	Pre	7	17	21	28	184
	501	Pre	7	15	22	29	183
	502	Pre	7	14	23	28	183
	503	Pre	7	14	21	28	181
	507	Pre	7	14	21	29	195
	548	Pre	7	14	21	28	271
Yellow Fever	Donor ID	Week 0	Week 1	Week 2	Week 3	Week 4	Week 8+
	4	Pre	8	15	20	28	184
	12	Pre	n/a	14	22	30	203
	558	1	n/a	15	22	29	218
	559	1	8	15	22	32	120
	565	1	9	16	23	30	60

Table 1: Yellow fever virus and vaccinia virus vaccine recipients. Blood sample timing for n=7 vaccinia virus vaccine recipients and n=5 yellow fever vaccine recipients are depicted in Table 1. Each row represents one donor with longitudinal samples collected at each day post vaccination. Columns show binning of sampling into weeks post vaccination.

post-YFV and VV vaccinations, which expanded and remained detectable in peripheral blood through week four post-vaccination (Figures 7A and 7B). The frequency of total T-bet⁺ B cells peaked around week four in the majority of individuals receiving either YFV (Figure 7C) or VV (Figure 7D) vaccination, suggesting that both vaccines stimulated a T-bet⁺ B cell response. The T-bet^{high}CD85j^{high} B cell frequency also peaked at week four in YFV-vaccinated individuals (Figure 7E), and expansion was preceded by expression of the activation marker Ki67 in this population at week two post vaccination (Figure 7F). While T-bet^{high}CD85j^{high} cell expansion was not detected in all donors during VV vaccination (Figure 7G), T-bet MFI in T-bet^{high}CD85j^{high} cells was dynamic and peaked between weeks three and four during both YFV and VV responses (Figures 7H and 7I, blue lines). These responses were not limited to a single surface Ig isotype, as T-bet expression increased in both IgM⁺ and class-switched T-bet^{high}CD85j^{high} cells (Figures 7J and 7K). Resting memory B cells (RM), which normally express low levels of T-bet (Figure 3B), displayed increased T-bet expression frequencies in several VV vaccine recipients (Figure 7M) but only in one YFV vaccine recipient (Figure 7L). This RM T-bet response involved only a small subset of cells, as RM population size did not increase (data not shown) and T-bet MFI in the total population remained static during both vaccinations (Figures 7H and 7I, black lines). Interestingly, T-bet levels in plasmablasts also increased following each vaccination (Figures 7N and 7O). Together, these findings indicate that human live viral vaccinations stimulate an acute T-bet⁺ B cell response and that the T-bet^{high}CD85j^{high} population may function as an early-responder during acute viral infections.

Figure 7

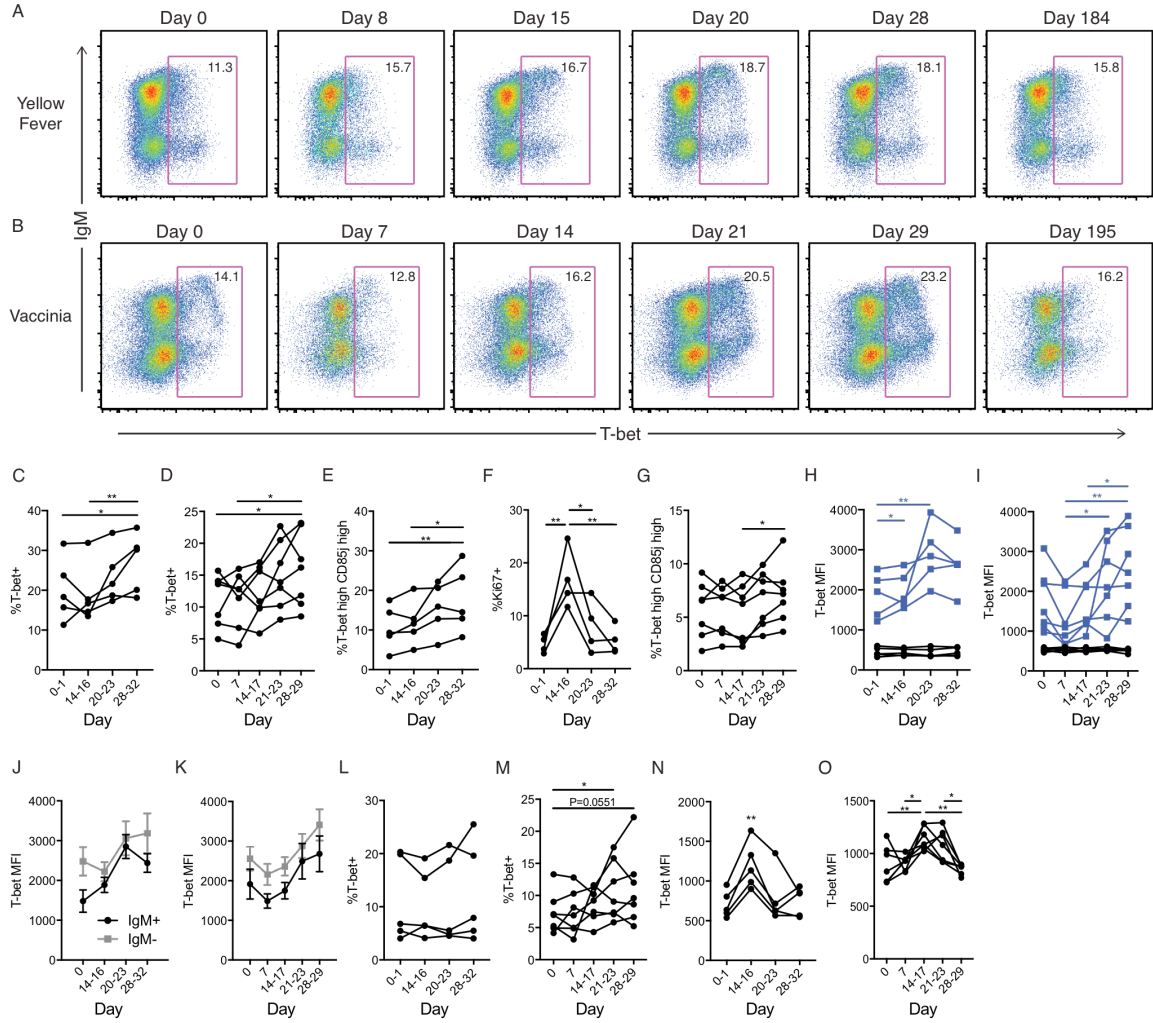


Figure 7: Longitudinal T-bet⁺ B cell dynamics in yellow fever virus-vaccinated or vaccinia virus-vaccinated individuals

Longitudinal T-bet expression in memory B cells of (A) a yellow fever (YFV) vaccinee and (B) a vaccinia (VV) vaccinee. Time of blood sampling post-vaccination is depicted. (C) T-bet expression frequency in total memory B cells of YFV vaccinees (n=5 for all YFV plots except F). Donor samples were binned to organize approximate weekly time points. (D) T-bet expression frequency of memory B cells in VV vaccinees (n=7 for all VV plots). (E) T-bet^{high}CD85^{high} cell frequency of total memory B cells in YFV vaccinees. (F) Ki67 expression frequency of T-bet^{high}CD85^{high} cells in YFV vaccinees (n=4). (G) T-bet^{high}CD85^{high} cell frequency of total memory B cells in VV vaccinees. (H) T-bet MFI of T-bet^{high}CD85^{high} cells (blue) and resting memory (RM) cells (black) in YFV vaccinees. No statistical differences were observed between RM time points. (I) T-bet MFI of T-bet^{high}CD85^{high} cells (blue) and resting memory (RM) cells (black) from VV

vaccinnees. No statistical differences were observed between RM time points. T-bet MFI of T-bet^{high}CD85j^{high} cells separated by IgM expression is depicted in (J) for YFV and (K) for VV; IgM⁺, black; IgM⁻ grey. Frequencies of RM cells expressing T-bet during YFV (L) and VV (M) responses are shown. No significant differences were observed in L between time points. (N) T-bet MFI of plasmablasts following YFV vaccination. (O) T-bet MFI of plasmablasts following VV vaccination. Statistical comparisons in C, D, E, F, G, H, I, L, M, N and O calculated using repeated measures one-way ANOVA with Tukey's multiple comparisons test. * denotes a P value $0.05 > P \geq 0.01$. ** denotes a P value $0.01 > P \geq 0.001$.

2.4 Discussion

The nature of the acute B cell response to human viruses remains poorly defined. To address this issue, we assessed the phenotype and dynamics of B cells expressing T-bet, a known regulator of Th1-type immunity across cell lineages, at rest and during acute human viral infections. We identified T-bet⁺ B cells in the peripheral blood memory compartment of healthy individuals and found an enrichment of antiviral antibody isotypes in this population. We further characterized T-bet^{low} and T-bet^{high} B cell subsets, the latter of which represents a distinct antigen-experienced population with an activated phenotype. These subsets further displayed disparate tissue localization profiles, as T-bet^{low} B cells were found in peripheral blood, thoracic duct fluid, and various lymphoid tissues, while T-bet^{high} B cells were restricted to the blood and spleen. B cell T-bet expression frequencies transiently increased in the blood in response to acute YFV and VV infections, a phenomenon attributable to T-bet induction across multiple B cell subsets and the specific activation and expansion of T-bet^{high}CD85j^{high} cells.

T-bet is considered to function as a master regulator of Th1-type immunity (100), and T-bet is known to promote antiviral B cell responses by driving IgG2a/c class-switching in mice (78, 110, 117, 118). Our findings suggest T-bet similarly regulates human B cells during acute Th1 immune responses, as we clearly demonstrate viral infection-induced T-bet expression in multiple human B cell subsets. Our results further suggest antibody isotype diversification as a key effect of this T-bet induction, as T-bet associates with the IgG1 and IgG3 isotypes in healthy individuals. However, we also observed activation of the T-bet⁺IgM⁺ population in response to acute YFV and VV infections; further experiments are necessary to determine if these IgM⁺ memory cells are particularly prone to IgG1 or IgG3 isotype switching, but this observation suggests additional antibody isotype-independent roles for T-bet in human B cells. T-bet may influence additional antibodies properties, as T-bet^{high}CD85j^{high} cells demonstrate elevated RNA transcript levels of *B4GALT3*, a galactosyltransferase that may alter antibody glycosylation (36), and *AICDA*, the enzyme critical for somatic hypermutation that leads to improved antibody affinity (37). T-bet may

also influence antibody-independent functions and properties: similar to mice (79, 117), T-bet likely regulates the expression of homing receptors CXCR3 and CD11c in human B cells, potentially granting specific tissue and inflammation homing properties to T-bet⁺ cells. Murine studies further suggest T-bet⁺ B cells in the spleen efficiently present antigen (63) and can be stimulated to produce the cytokine IFN γ (65).

Our study identified a heterogeneous T-bet-expressing B cell population in human blood comprised of two main subsets: T-bet^{high}CD85j^{high} cells (CD21⁺T-bet^{high}CD85j^{high}) and T-bet-expressing RM cells (CD21⁺CD27⁺T-bet^{low}). T-bet^{high}CD85j^{high} cells expand in response to acute human viral infections (YFV and VV vaccinations) and closely resemble age-associated B cells, a T-bet⁺ population in mice that accumulates with age, produces pathogenic autoantibodies, and is critical for murine antiviral responses (114, 115, 117, 118). In contrast to T-bet^{high}CD85j^{high} cells, we did not observe significant expansion or activation of T-bet^{low} cells in most individuals following YFV or VV vaccination, suggesting this subset does not play an active role during acute antiviral B cell responses. While the origin and functions of T-bet^{low} cells remain unclear, they may represent quiescent descendants of T-bet^{high}CD85j^{high} cells that maintain long-lived antiviral memory, as acutely-activated virus-specific B cell clones have been shown to seed the RM population following resolution of acute infection (159).

We further identified differential circulatory abilities and tissue distributions of T-bet^{high} and T-bet^{low} cells. The T-bet^{low} population was found in all assessed tissues, suggesting peripheral blood T-bet^{low} cells can enter into lymphoid organs, exit via the efferent lymph, and travel to other lymphoid tissues or back into the blood via the thoracic duct. It is unclear whether these cells remain in lymphoid tissue for extended periods of time or pass through transiently. Conversely, we identified T-bet^{high} cells in peripheral blood and the spleen but could not detect them in lymph nodes, tonsil, or thoracic duct fluid, suggesting T-bet^{high} cells do not traffic into lymph nodes and do not egress from tissues in the absence of an immune response. The relationship between splenic CD21⁺T-bet⁺ cells and the peripheral T-bet^{high} and T-bet^{low} subsets is

unclear, but the sheer size of the splenic CD21⁺T-bet⁺ population suggests portions of these cells are spleen-resident.

In summary, our study identifies T-bet^{high} B cells as a novel viral infection-responsive subset and suggests T-bet as an important mechanism regulating humoral antiviral immunity in humans. T-bet^{high} B cells' rapid expansion during acute viral infections is reminiscent of effector T cells and suggests they participate in the immune response by generating IgG1 and IgG3 isotype antibodies and homing to sites of inflammation. In combination with T-bet^{high} B cells' responsiveness to viral infection, the previously reported outgrowth of T-bet-expressing B cells during inflammatory conditions (120, 121, 162, 165, 166) suggests this population may be broadly sensitive to inflammatory signals. Future studies should assess the direct contribution of T-bet^{high} B cells to antigen-specific responses and investigate relationships between disparate T-bet⁺ tissue B cell subsets to fully define the human T-bet⁺ B cell compartment's immunological niche.

CHAPTER 3

T-BET-EXPRESSING B LYMPHOCYTES ARE INDUCED BY HIV INFECTION AND DOMINATE THE GP140-SPECIFIC B CELL RESPONSE

3.1 Summary

Current HIV vaccine efforts seek to induce protective antibodies, but the development and regulation of the B cell response to HIV remain poorly understood. Here, we assessed the role of T-bet-expressing B cells, a population critical for murine antiviral responses, during HIV infection. T-bet⁺ B cells expanded during early acute HIV infection, were maintained at excessive levels in chronic viremic infection, and associated with increased serum and cell-associated IgG1 and IgG3 expression. Viral control was associated with contracting frequencies of T-bet^{high} B cells, but T-bet^{low} B cells remained expanded in many aviremic donors. siRNA knockdown studies further suggested a role for T-bet in the regulation of activation-induced cytidine deaminase RNA expression. The HIV gp140-specific B cell response was dominated by T-bet-expressing memory B cells, and we observed a concomitant biasing of gp140-specific serum immunoglobulin to the IgG1 isotype. These findings suggest sustained T-bet induction by HIV infection alters the isotype landscape of the B cell compartment and drives expansion and maintenance of a large T-bet⁺ B cell population that coordinates the HIV Env-specific humoral response.

3.2 Introduction

The HIV pandemic persists as one of the most significant global health problems (131). While antiretroviral therapy has greatly improved mortality rates, a preventative vaccine remains necessary to curtail the spread of HIV (175). Efforts have shifted to rational-based vaccine design, requiring an in-depth analysis of immune responses to identify and stimulate protective immunological correlates (144). Recent isolation and characterization of many naturally occurring HIV-specific broadly neutralizing antibodies demonstrates the capacity of humans to generate a potentially protective humoral response (176), but the B cells and mechanisms regulating humoral immunity to HIV remain poorly characterized. An improved understanding of the B cell response will identify interventional targets and inform rational vaccine design for HIV and other viruses for which broadly effective vaccines do not exist.

HIV infection is characterized by excessive viral replication and inflammation that induce a strong virus-specific humoral response and promote polyclonal B cell stimulation (54, 147). This B cell hyperactivation likely contributes to previously described B cell subset alterations in chronically infected individuals (147). The memory B cell compartment is particularly impacted by HIV, with decreased resting memory B cell numbers and an expansion of activated and atypical memory B cells that lack expression of the complement receptor CD21 (150, 151). It was previously demonstrated that HIV-specific responses are over-represented in CD21⁻ memory B cells in viremic individuals (155), but the factors regulating HIV-specific B cells are unclear.

B cells expressing the transcription factor T-bet, a broad regulator of Th1-type immune responses, are critical for controlling murine chronic viral infections (117, 118). We recently identified T-bet-expressing B cells in human peripheral blood that are enriched for IgG1 and IgG3, two antibody isotypes that efficiently mediate antiviral effector functions (Chapter 2). T-bet^{high} cells, a subset of this population, are highly activated and transiently expand in response to vaccination with diverse replicating viruses. These properties suggest T-bet⁺ B cells participate in human antiviral responses, but their role during HIV infection is unknown.

In this study we examine HIV-induced T-bet expression in B cells, the consequences of this induction, and the involvement of T-bet⁺ B cells in the HIV immune response. We identified rapid activation and expansion of T-bet⁺ B cells and increased T-bet expression levels in plasmablasts during early acute HIV infection. Following the resolution of acute infection, the T-bet⁺ memory B cell population was maintained at a high frequency into chronic HIV infection and correlated with increased expression and secretion of IgG1 and IgG3 antibodies. This T-bet⁺ B cell population maintains the anti-gp140 B cell response and, via the expression of T-bet, likely biases gp140-specific antibodies to the IgG1 isotype. Our data identify T-bet⁺ B cells as responders during both acute and chronic HIV infection and suggest this population orchestrates the humoral immune response to HIV.

3.3 Results

3.3.1 Acute HIV infection induces T-bet⁺ B cell population expansion

In our previous study (Chapter 2) we assessed B cell dynamics during YFV and VV vaccinations, which represent relatively controlled, low inflammation infections. We therefore investigated whether T-bet⁺ B cells similarly expand during acute HIV infection, which is characterized by excessive inflammation and immune pathology. To assess this, we obtained longitudinal peripheral blood mononuclear cell samples from high-risk HIV seronegative individuals who subsequently became infected with HIV from the U.S. Military HIV Research Program RV217 early capture HIV cohort (177). We analyzed T-bet expression in total memory B cells (Figure 2A) from pre-infection, early acute, and early chronic infection time points (Table 2). During early acute infection (days 1-21 after first positive HIV RNA test), six out of seven individuals demonstrated a rapid increase in the frequency of T-bet-expressing memory B cells, supporting the role of these cells as part of an early antiviral response (Figure 8A and 8B). Similar to YFV

Figure 8

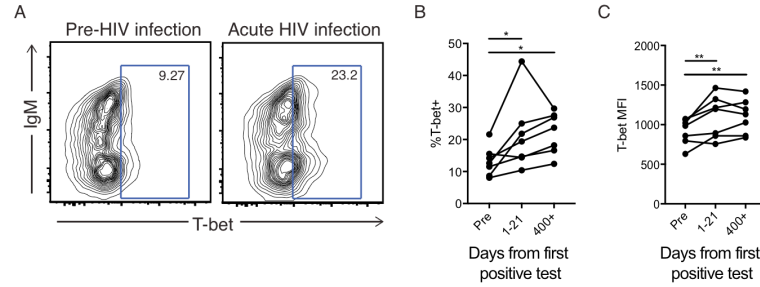


Figure 8: Longitudinal T-bet⁺ B cell dynamics in acutely HIV-infected individuals

(A) T-bet expression in memory B cells of an acutely HIV-infected individual before infection and shortly after peak of viremia. **(B)** Memory B cell T-bet expression frequency from 7-donor cohort of acutely HIV-infected individuals at pre-infection, acute, and chronic infection time points. **(C)** T-bet MFI of plasmablasts at pre-infection, acute, and chronic infection time points. Note that intracellular T-bet staining for these samples was performed using BD Cytofix/Cytoperm Kit (Cat. # 554722). Statistical comparisons in B and C calculated using repeated measures one-way ANOVA with Tukey's multiple comparisons test. * denotes a P value $0.05 > P \geq 0.01$. ** denotes a P value $0.01 > P \geq 0.001$.

Donor ID	Day relative to first pos. test	VL (copies/ml)	CD4 count (cells/ul)
10374	-194	n/a	n/a
	21	10965	775
	407	17783	446
30507	-289	n/a	n/a
	17	1122	1574
	413	7585	n/a
40067	-467	n/a	n/a
	1	229087	565
	403	23988	425
40134	-69	n/a	n/a
	2	2454709	435
	433	13804	n/a
40250	-173	n/a	n/a
	14	4677351	522
	420	16596	461
40283	-478	n/a	n/a
	1	398107	376
	425	50199	n/a
40353	-41	n/a	n/a
	14	162181	574
	415	3388	448

Table 2: RV217 Early Capture HIV cohort. Longitudinal samples were obtained at pre-infection, acute, and chronic infection time points for n=7 donors, with the collection date listed as day relative to first HIV-positive RNA test. Viral load and CD4 counts are listed where available. Individuals were therapy-naïve at the time of sampling.

and VV vaccinations (Chapter 2), we also observed increased T-bet MFI in plasmablasts at this acute time point (Figure 8C). These six individuals remained viremic into early chronic infection and concomitantly maintained elevated T-bet levels, suggesting a relationship between continued viral replication and maintenance of this expanded population (Figures 8B and 8C).

3.3.2 Chronic HIV infection maintains an expanded T-bet⁺ B cell population

Having observed the rapid expansion and sustained expression of T-bet⁺ B cells in acute to early chronic HIV infection, we next examined T-bet expression in memory B cells from chronically infected HIV⁺ cohorts with different viral loads: viremic HIV progressors (progressors; VL>10,000 copies/ml), viremic controllers (VC; VL 41-1800 copies/ml), elite controllers (EC; VL <40 copies/ml), aviremic individuals on antiretroviral therapy (ART; VL<40 copies/ml), and HIV-negative controls (Tables 3 and 4). We identified a significant expansion of T-bet⁺ memory B cells in HIV-infected progressors and viremic controllers compared to healthy controls (Figure 9A and 9B). In progressors, individuals with the highest viremia, this expansion was comprised almost entirely of T-bet^{high}CD85j^{high} B cells (Figure 9C) and coincided with higher expression levels (MFI) of T-bet (Figure 9D). Absence of detectable viremia in EC and ART was associated with decreased T-bet⁺ B cell population size in many donors compared to viremic individuals (Figure 9B), which could be explained in part by diminished T-bet^{high}CD85j^{high} cell expansion in these cohorts (Figure 9E). We also observed a trend toward elevated T-bet expression frequencies in RM B cells in all HIV⁺ cohorts (Figure 9F); however, similar to our findings in HIV-negative donors (Chapter 2, Figure 3C), RM cell T-bet expression levels (MFI) remained low (data not shown). Lastly, we observed increased T-bet MFI in plasmablasts of progressors and viremic controllers (Figure 9G), suggesting together with our acute infection findings that viral replication also promotes T-bet expression in plasmablasts. Together, our data indicate chronic HIV viremia drives T-bet expression in both memory B cells and plasmablasts and maintains the expansion of the T-bet^{high}CD85j^{high} subset.

Donor ID	Group	VL	CD4 ct.
110029	ART	Undetectable	800
110564	ART	Undetectable	1091
110595	ART	Undetectable	702
110600	ART	Undetectable	828
110657	ART	Undetectable	1188
110869	ART	Undetectable	679
110870	ART	Undetectable	693
110887	ART	Undetectable	689
111031	ART	Undetectable	338
111096	ART	Undetectable	686
110015	ART	Undetectable	698
110029	ART	Undetectable	624
110043	ART	Undetectable	564
110054	ART	Undetectable	644
110163	ART	Undetectable	640
110206	ART	Undetectable	423
110285	ART	Undetectable	481
110313	ART	Undetectable	804
110334	ART	Undetectable	431
110348	ART	Undetectable	1438
110351	ART	Undetectable	382
110399	Prog.	141958	70
110670	Prog.	198018	248
110939	Prog.	97259	250
110967	Prog.	224078	8
111005	Prog.	41679	221
111031	Prog.	500000	90
111081	Prog.	280428	222
111089	Prog.	339292	377
111112	Prog.	466000	5
111154	Prog.	205176	8
110187	Prog.	14573	393
110245	Prog.	10210	216
110360	Prog.	25320	349
110554	Prog.	41600	777
110622	Prog.	57465	471
110673	Prog.	28000	387
110763	Prog.	25876	270
110979	Prog.	*Undetectable	1323
111056	Prog.	145654	494
111083	Prog.	17284	323
111117	Prog.	33940	364
130236	Prog.	28888	632
130636	Prog.	35160	323

130716	Prog.	99562	19
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Table 3: University of Pennsylvania Center for AIDS Research donor samples. Viral loads and CD4 counts for chronically infected HIV+ cohorts, including antiretroviral therapy (ART)-treated and ART-naïve, viremic individuals (Progressors), are depicted. The individual marked with * had undetectable viremia at the time of collection but had a viral load of 18,083 at the subsequent clinic visit 20 months later.

Donor ID	Group	VL	CD4 ct.
1161	VC	360	1185
1176	EC	<40	757
1203	EC	<40	890
1204	EC	<40	1032
1215	VC	1183	1747
1247	VC	1102	591
1277	VC	76.3	1074
1367	EC	<40	566
1446	VC	305	1086
1448	VC	1400	485
1508	VC	201	427
1526	EC	<40	531
1527	EC	<40	1085
1541	VC	1589	1174
1545	VC	182	625
1555	EC	<40	613
1581	EC	<40	1227
1637	VC	676	909
3604	EC	<40	912
4014	EC	<40	982

Table 4: University of California San Francisco SCOPE cohort. Viral loads and CD4 counts for viremic controllers (VL 41-1800 copies/ml) and elite controllers (VL <40 copies/ml) are depicted. All individuals were therapy-naïve at time of sampling.

As our data implicate T-bet^{high}CD85j^{high} cells as participants in antiviral responses, and members of this population have been described as exhausted during chronic HIV infection (151), we next asked whether chronic HIV infection might selectively induce transcriptional changes in T-bet^{high}CD85j^{high} cells and therefore potentially impact their functionality. We compared RNA transcript levels of our 91-member panel (Chapter 2, Figure 4A) in T-bet^{high}CD85j^{high} cells between HIV-negative individuals (n=4) and progressors (n=5) and found that T-bet^{high}CD85j^{high} cells appear largely similar regardless of HIV infection status (Figure 9H). These findings suggest that, while viremic HIV infection greatly expands and maintains the T-bet^{high}CD85j^{high} population, it does not overtly alter the transcriptional environment of these cells during the course of infection.

Figure 9

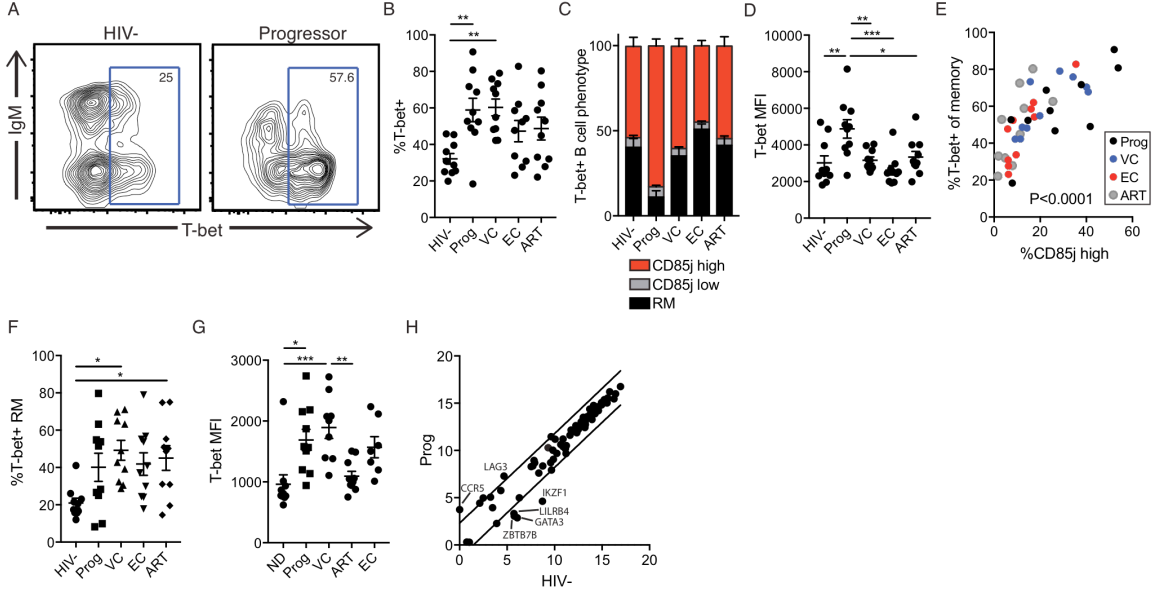


Figure 9: T-bet expression in B cells and RNA transcript levels in T-bet^{high}CD85j^{high} cells during HIV infection.

(A) T-bet expression in memory B cells of a representative HIV-negative donor and an HIV⁺ chronic progressor (progressor). (B) T-bet expression frequency of memory B cells in HIV-negative, HIV⁺ progressor, HIV⁺ viremic controller (VC), HIV⁺ elite controller (EC), and HIV⁺ antiretroviral therapy-treated donors (ART); n=10 donors per group. (C) Phenotype of T-bet⁺ B cell population from each cohort in B. Bars represent standard error. (D) T-bet MFI of total T-bet⁺ cells from each cohort. (E) Correlation of total T-bet expression and T-bet^{high}CD85j^{high} cell frequencies of memory B cells. Four HIV⁺ cohorts are represented by different colors: Progressors, Prog, black; Viremic controllers, VC, blue; Elite controllers, EC, red; Aviremic individuals on antiretroviral therapy, ART, gray. Statistics were calculated using Spearman correlation. (F) Frequency of RM cells expressing T-bet by cohort. (G) T-bet MFI of plasmablasts by cohort. (H) Comparison of transcript expression levels for 91 gene targets between healthy donors (n=4) and progressors (n=5). Each dot represents the mean expression value calculated for HIV-negative donors (x value) and progressors (y value). Lines represent 90% prediction bands of calculated linear regression. Statistical comparisons in B, D, F, and G calculated using one-way ANOVA with Tukey's multiple comparisons test. * denotes a P value 0.05 > P ≥ 0.01. ** denotes a P value 0.01 > P ≥ 0.001. *** denotes a P value < 0.001.

3.3.3 Sustained T-bet expression in B cells alters the antibody isotype repertoire

As T-bet is associated with specific IgG isotypes (Chapter 2, Figure 2E), we hypothesized that the maintenance of elevated T-bet expression in B cells and plasmablasts during HIV infection might alter memory B cell surface IgG isotype distribution and serum antibody repertoires in these individuals. We therefore analyzed memory B cells from HIV-negative individuals and progressors and found increased frequencies of IgG1- and IgG3-expressing memory B cells during HIV infection (Figure 10A). To explore possible downstream effects on secreted antibody, we next assessed the isotypes of total serum antibody from progressors and age/ethnicity matched HIV-negative controls and found approximately 2-fold increases in titers of IgG1 and IgG3 in progressors as compared to controls (Figure 10B). IgG1 titers in viremic subjects positively correlated with the frequency of T-bet⁺ memory B cells (though this did not reach statistical significance due to a single outlier), while IgG2, IgG3, IgG4, and IgA titers displayed no relationship (Figure 10C). IgM titers also positively correlated with T-bet expression frequency (Figure 10C), suggesting that activation events in early memory B cell differentiation may be skewed by T-bet expression. In summary, HIV drives the expansion and maintenance of T-bet⁺ memory B cells and plasmablasts, which correlate with an overrepresentation of surface-expressed and soluble IgG1 and IgG3 during viremic infection.

We next set out to examine the relationship between T-bet activation-induced cytidine deaminase (AID, or *AICDA* as transcript), the B cell-specific enzyme that is required for antibody isotype switching (37). Within the peripheral B cell compartment, *AICDA* expression is highest in T-bet^{high}CD85j^{high} B cells, and transcript levels remain elevated in this subset during chronic HIV infection (Tables 3 and 5; Figure 10D). To examine the potential regulation of *AICDA* transcript expression by T-bet, we performed siRNA-mediated downregulation of T-bet in primary CD21⁺CD27⁺ B cells from progressors with high T-bet expression frequencies in this subset (mean 93.6% T-bet⁺, range 81.2% to 97.6%; Figure 10E), as previously described (178); cells consistently retained their CD85j^{high} phenotype following nucleofection (Figure 10F). CD21⁺CD27⁺

Figure 10

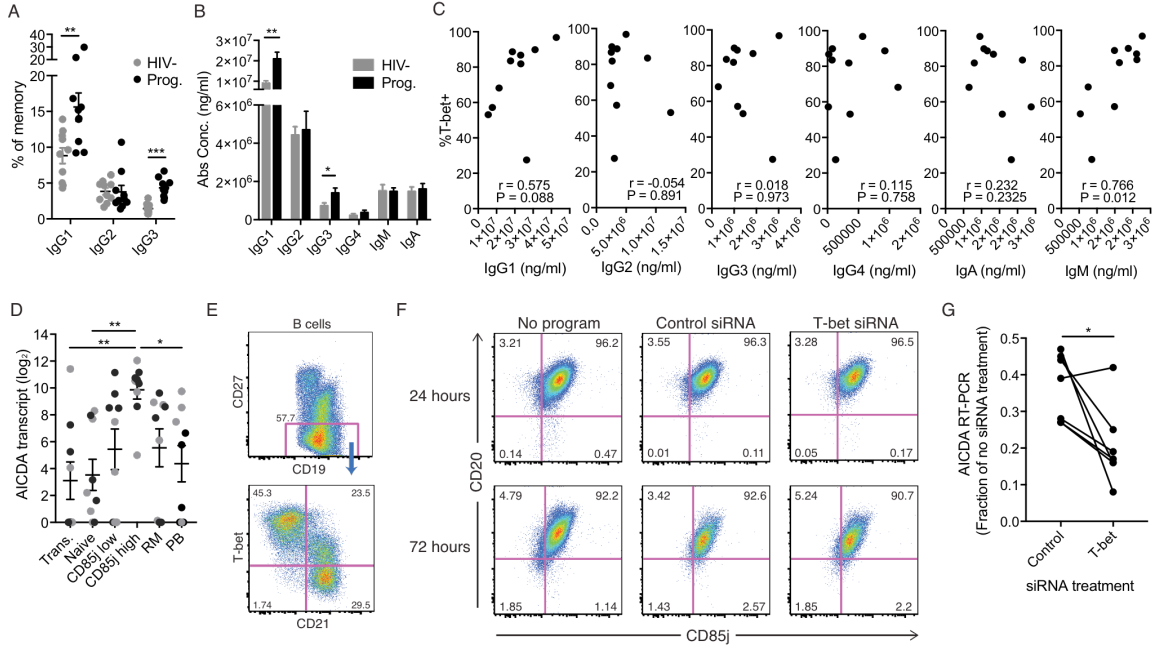


Figure 10: Antibody isotype expression levels, correlations with T-bet, and siRNA T-bet knockdown in B cells of progressors.

(A) Frequency of IgG isotype-expressing B cells within total memory B cell compartment of HIV-negative individuals and progressors (HIV⁺) (n=10 donors per group). Statistics in A and B calculated using unpaired t-test. (B) Absolute concentration of total serum antibodies by isotype from progressor (HIV⁺) and age/ethnicity-matched HIV-negative cohorts (n=10 donors per group). (C) Correlations between T-bet expression frequency in memory B cells and serum antibody titers by isotype (ng/ml). Statistics in C calculated using Spearman correlation. (D) *AICDA* transcript expression (log₂ units) of HIV-negative donors (grey; n=4) and progressors (black; n=5) per B cell subset. Trans., transitional; RM, resting memory; PB, plasmablast. Each dot represents transcript expression from one individual. Statistical comparison calculated using repeated measures ANOVA with Tukey's multiple comparisons. (E) Expression of CD21 and T-bet within CD27⁺ B cells from a representative progressor used for CD21⁺CD27⁺ B cell sorting. (F) CD85j expression by sorted CD21⁺CD27⁺ B cells at 24 and 72 hours post-nucleofection with either control or T-bet siRNA, compared to non-nucleofected sample. (G) RT-PCR of *AICDA* transcript in CD21⁺CD27⁺ B cells of progressors transfected with control or T-bet siRNA. Values represent *AICDA* transcript level as a fraction of no siRNA treatment condition. Statistical comparison in G calculated using paired t-test. * denotes a P value 0.05 > P ≥ 0.01; ** denotes a P value 0.01 > P ≥ 0.001.

Donor ID	Cohort	Duration	Status	VL	CD4 ct.
OM10	Toronto	>1 year	Chronic	22415	132
OM17	Toronto	<6 months	Early	440000	660
OM255	Toronto	<6 months	Early	18322	370
OM284	Toronto	<3 months	Acute	170693	430
OM5007	Toronto	<3 months	Acute	24019	440
OM5031	Toronto	<1 year	Chronic	13537	330
OM5037	Toronto	<3 months	Acute	13948	780
OM5062	Toronto	>6 months	Chronic	187361	410
OM5220	Toronto	<3 months	Acute	10640	470
OM5271	Toronto	<3 months	Acute	25947	320
P09586	UPenn CFAR	2.5 months	Acute	23990	446
P09588	UPenn CFAR	>1 year	Chronic	80000	<500
P09603	UPenn CFAR	67 days	Acute	442034	922
P09636	UPenn CFAR	6 months	Early	43374	605

Table 5: Acute and chronic HIV samples from University of Toronto and University of Pennsylvania CFAR cohorts. Viral loads and CD4 count for University of Toronto and University of Pennsylvania cohorts are depicted. The listed duration of infection is estimated: Acute samples are estimated to have been collected <3 months since infection; Early samples are between 3-6 months since infection; Chronic samples are from greater than 6 months since infection. All individuals were therapy-naïve at time of sampling.

B cells were chosen as a representation of the T-bet^{high}CD85j^{high} population because they can be identified and sorted via negative selection, avoiding potential ligation effects of CD85j-specific antibodies. T-bet siRNA knockdown reduced levels of *AICDA* by 44% compared to control siRNA, indicating that T-bet at least partially regulates expression of *AICDA* in this population and may promote class switching via this regulation (Figure 10G).

3.3.4 HIV infection primarily drives a T-bet⁺ B cell response to HIV envelope protein gp140

Finally, we examined the relationship between T-bet⁺ B cells and the B cell response to HIV, focusing on the Env-specific response since this antigen is the relevant antibody target during HIV infection (54). Using a group M consensus sequence-derived gp140 protein to capture soluble antibody in an ELISA format, we analyzed the isotype repertoire of the gp140-specific antibody in serum of progressors (Table 3) and found that, in agreement with previous studies (179-184), Env-specific responses are dominated by IgG1 isotype antibodies (Figure 11A), the most frequent IgG isotype associated with T-bet-expressing B cells. To directly connect this expanded Env-specific IgG1 usage to the cellular component of the response, we used gp140 protein fluorophore probes to identify gp140-specific B cells via flow cytometry, as previously described ((152); Figure 11B). Progressors, several of which were estimated to be in the early acute phase of infection, at <3 months HIV⁺ (see Table 5), demonstrated a mean gp140-specific B cell frequency of 0.86% of class-switched B cells in peripheral blood, and this frequency was diminished in HIV⁺ cohorts with lower viremia (Figure 11C). Interestingly, nearly all gp140-specific B cells expressed T-bet, regardless of the HIV⁺ donor's viral load (Tables 4 and 5; Figures 11D and 11E). gp140-specific B cells from HIV viremic individuals consistently demonstrated a T-bet^{high}CD85j^{high} phenotype, while the gp140-specific B cell response in subjects with lower or controlled viremia was composed of both T-bet^{high}CD85j^{high} cells and T-bet^{low} RM B cells (Figure 11F and data not shown). Taken together, these findings demonstrate that the humoral immune response to HIV Env is specifically coordinated by the T-bet⁺ memory B cell population and

suggests induction of this transcription factor as a mechanism driving the predominantly IgG1-based Ig response to the virus.

Figure 11

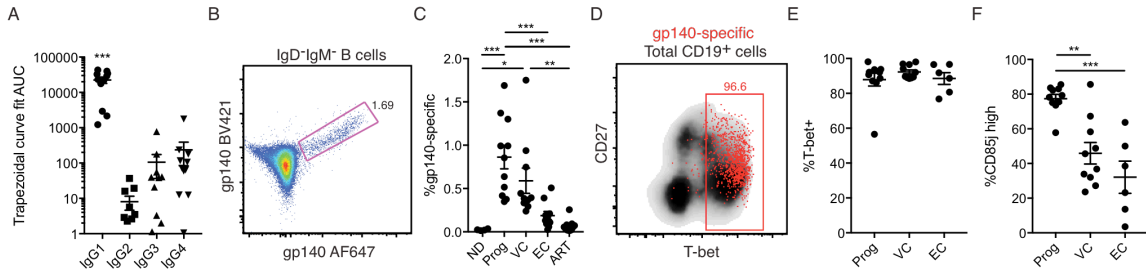


Figure 11: gp140-specific memory B cell phenotypes and serum Ig isotypes.

(A) gp140-specific serum Ig titers by isotype from progressors (n=10). Titers were normalized using age/ethnicity matched controls. Statistical comparison calculated using repeated measures one-way ANOVA with Tukey's multiple comparisons test. (B) Representative staining of gp140-specific class switched (IgD⁺IgM⁻) memory B cells from a progressor. (C) Frequency of gp140-specific cells within total class-switched memory B cells by cohort. n=10 for all cohorts except progressors (n=11). (D) T-bet expression of gp140-specific B cells (red) and total B cells (black) from a representative progressor. (E) Frequency of T-bet expression within gp140-specific cells from HIV⁺ cohorts (n=10 progressors, n=10 VC, n=6 EC). All donors with <40 gp140-specific cells (including all ART individuals) were excluded from analyses in E and F. No statistical differences were observed between donor groups. (F) Frequency of gp140-specific cells with T-bet^{high}CD85^{high} phenotype by cohort. For C, E, and F, statistical comparisons were calculated using one-way ANOVA with Tukey's multiple comparisons test. * denotes a P value 0.05 > P ≥ 0.01; ** denotes a P value 0.01 > P ≥ 0.001. *** denotes a P value < 0.001.

3.4 Discussion

The identity of the cells and regulatory mechanisms orchestrating the humoral HIV response has remained unclear. We tested the hypothesis that T-bet⁺ B cells, which expand during acute resolving viral infections and express antiviral antibody isotypes, contribute to the HIV response. Similar to YFV and VV vaccinations, acute HIV infection elicits an expansion of T-bet⁺ B cells; however, unlike the transient nature of acute YFV and VV responses, chronic HIV viremia maintains elevated T-bet⁺ B cell frequencies that are associated with increased serum IgG1 and IgG3 titers. These T-bet⁺ cells dominate the HIV Env-specific B cell response and likely skew the anti-Env serum antibody repertoire to the IgG1 isotype.

In agreement with our previous study of HIV⁻ donors (Chapter 2), our current observations of increased IgG1⁺ and IgG3⁺ memory B cell frequencies in viremic HIV⁺ donors maintaining elevated T-bet expression further support T-bet as a mechanism promoting IgG1 and IgG3 isotype switching. Memory B cells do not actively secrete antibody; however, viremic HIV⁺ individuals also displayed increased T-bet MFI in plasmablasts, increased total IgG1 and IgG3 serum titers, and an IgG1-dominated Env-specific serum antibody response, suggesting IgG1⁺ and IgG3⁺ antibody-secreting cells readily differentiate from or share a similar origin with IgG1⁺ and IgG3⁺ memory B cells. It is unclear why IgG3 antibodies are not more prevalent within the anti-Env antibody repertoire during chronic HIV infection when T-bet expression is high, but IgG3 levels have been demonstrated to peak early during HIV infection and wane over time (54). It is possible that sustained HIV viremia drives recurrent germinal center re-entry and isotype switching of memory B cells, which could promote splicing out of the proximally located $\gamma 3$ gene from the constant region locus as the infection progresses; indeed, a recent study suggests class-switching of IgG3⁺ B cells would likely give rise to either IgG1⁺ or IgG2⁺ cells (185). Together, our findings suggest T-bet expression in B cells coordinates the IgG1-dominated anti-Env response and likely regulates antibody isotype selection during other human viral infections.

AID expression is tightly controlled by multiple factors (38), but our siRNA T-bet knockdown experiments suggest that T-bet may regulate IgG1 and IgG3 class switching in part by promoting *AICDA* expression, although it is unclear whether this occurs by direct or indirect mechanisms. In addition to isotype switching, AID is required for somatic hypermutation (37), the diversification of antibody binding sites. Therefore, by regulating *AICDA*, T-bet may also promote antibody mutation, and a recent murine study supports this notion by demonstrating significant mutation rates in BCRs from T-bet-expressing B cells (186). However, antibody mutation may be impaired in subsets of T-bet⁺ B cells during progressive HIV infection, as HIV-specific antibodies derived from TLM cells demonstrated diminished mutation levels compared to RM cell-derived antibodies (187). Future studies should examine whether relatively low TLM antibody mutation rates are typical (i.e. observed in healthy individuals mounting successful humoral responses) or are instead reflective of immunopathology specific to chronic HIV infection; in support of the latter possibility, malaria-specific antibody derived from TLM-phenotype B cells displayed higher mutation rates than those derived from RM cell counterparts (188).

HIV⁺ cohort analyses identified involvement of both T-bet^{high} (CD21⁺T-bet^{high}CD85j^{high}) and T-bet^{low} (CD21⁺CD27⁺T-bet^{low}) B cell subsets in the HIV response, with potentially divergent roles. T-bet^{high} cells were induced early and maintained the Env-specific B cell response during chronic viremic HIV infection, with a reduction in T-bet^{high} population size following natural or therapy-mediated control of viremia. Combined with our YFV and VV observations (Chapter 2), these findings suggest T-bet^{high} cells are a major component of acute and chronic antiviral B cell responses and, in the setting of HIV, are responsive at a population level to modulation of viremia and immune activation. Conversely, reduction of viremia during chronic HIV infection did not affect expanded T-bet^{low} RM cell population size, suggesting these cells do not depend on viral replication for their maintenance. Viral control was also associated with a greater representation of T-bet^{low} cells within the Env-specific pool, suggesting the Env-specific response is mediated by different T-bet⁺ B cell subsets depending on disease course: T-bet^{high} cells during acute infection

and settings of high viremia, and T-bet^{low} B cells in the context of controlled but persistent low level viremia.

We and others have previously demonstrated an expansion of CD21⁻ B cell subsets induced by Th1-type human infections, including HIV, Malaria, and Hepatitis C (120, 150, 151, 160, 161, 165, 189, 190), and expansion of a clonal CD21⁻ subset has been described during Hepatitis B- and Hepatitis C-associated mixed cryoglobulinemia (191-193). Interestingly, maintenance of expanded CD21⁻ cells in each of these infections appears to be dependent upon pathogen load (150, 151, 189, 194-196). Our current findings, combined with the similar CD21⁻ B cell phenotypes described in these studies, suggest that CD21⁻ B cell expansion associated with these various Th1-type infections may in part be explained by infection-induced T-bet expression and expansion of T-bet^{high}CD85j^{high} cells. In support of this hypothesis, murine experiments suggest the T-bet⁺ B cell phenotype is induced by concerted actions of pathogen-derived nucleic acids (TLR7 or TLR9 stimulation; (113-117, 197)) and cytokines produced during Th1-type infections (IFN γ and IL-21; (65, 78, 116)) on B cells. Still, additional studies are necessary to determine the relationship between acute viral infection-induced T-bet^{high}CD85j^{high} cells and these chronic infection expanded CD21⁻ B cell subsets. Interestingly, observational human studies further demonstrate similar CD21⁻ B cell expansion during various autoimmune diseases (73, 119, 121, 162, 163, 166), and murine models indicate TLR sensing can stimulate T-bet expression to drive expansion of autoantibody-producing B cells (78, 115), suggesting CD21⁻ T-bet⁺ B cells may also play a role in human autoimmune diseases.

While our YFV and VV acute infection observations (Chapter 2) suggested T-bet⁺ B cells generally represent a component of the antiviral B cell response, we specifically demonstrated that T-bet⁺ B cells compose nearly the entire detectable peripheral B cell HIV Env memory response, beginning at least during early (<3 months HIV⁺) infection. As such, most gp140-specific antibodies likely derive from descendants of the T-bet⁺ B cell population or share a similar T-bet-regulated origin in both progressors and individuals controlling viremia. This likely also includes HIV-specific broadly neutralizing antibodies, but requires further assessment as

previous studies that isolated broadly neutralizing antibodies from B cells generally did not consider the nature of the cells. An important question remaining is the degree to which T-bet⁺ B cells and derived antibodies contribute to control of viremia during HIV and other viral infections. Indeed, while a murine study demonstrated the requirement of T-bet⁺ B cells to control chronic LCMV viremia to low levels (118), the role of B cells and immunoglobulin in ongoing control of HIV/SIV remains controversial (198-202). However, it is highly likely that an efficacious HIV vaccine will need to induce HIV Env-specific broadly neutralizing antibodies. As such, our results directly demonstrate the importance of T-bet-expressing B cells to the HIV-specific humoral response.

CHAPTER 4

CONCLUSIONS, IMPLICATIONS, AND FUTURE DIRECTIONS

Introduction

A more complete understanding of B cell biology is critical for the development of vaccines and other interventions to improve humoral immunity to viruses, particularly in the case of HIV infection. As such, we sought to better define the cellular members and mechanisms that directly promote human antiviral responses and help to maintain humoral memory. In chapters 2 and 3 of this dissertation, we identified a previously unappreciated T-bet-expressing B cell population and described a number of characteristics, including cell phenotypes, population dynamics, and tissue localization, that identify human T-bet⁺ B cells as a unique viral infection-responsive subset. Our findings provide critical insights into the T-bet-regulated generation of humoral viral responses, identify T-bet⁺ B cells as the specific mediators of the HIV Env response, and raise a number of questions that provide a framework for further dissecting the biology of human T-bet⁺ B cells. The conclusions, implications, and future directions of this dissertation's key findings will be discussed below.

T-bet as a mediator of Th1 immunity in human B cells

Recent murine studies demonstrate a role for T-bet-expressing B cells in viral control (117, 118), but the mechanisms driving antiviral immunity in human B cells are poorly understood. Our observed induction of T-bet expression across the B cell compartment during diverse acute viral infections (low versus high viremia and inflammation; RNA versus DNA viruses) and chronic HIV infection strongly suggests a role for T-bet as a critical regulator of antiviral humoral immunity in humans. T-bet's association with IgG1 and IgG3 antibody isotypes in both HIV⁻ individuals and those mounting a substantial HIV immune response identifies promotion of class switching as an important function mediated by the transcription factor to combat viruses. Our findings further

suggest that T-bet expression promotes the differentiation of a unique activated B cell subset to mediate the acute antiviral response and maintains a resting memory pool prepared to initiate secondary immune responses (discussed further below). Via these diverse mechanisms, T-bet appears to broadly coordinate humoral antiviral immunity in humans.

The substantial induction of T-bet during acute viral responses, particularly in the T-bet^{high} subset, suggests T-bet may act as a master regulator of Th1-type B cell development. However, it is unclear whether T-bet is sufficient to drive Th1 B cell development in humans or is simply a marker of this program, acting as a minor player within a larger transcriptional network. In support of the former possibility, T-bet controls a broad transcriptional program in T cells and is required for both Th1 CD4⁺ T cell development and the generation of IgG2a⁺ B cell responses in mice (78, 99). Our findings suggest human B cell development mirrors that of CD4⁺ T helper cell differentiation (i.e. Th1 vs. Th2) in which cytokines and other signals guide specific differentiation pathways at the expense of others (203). Subsequent analyses of T-bet⁺ B cell differentiation, polarization, and plasticity will be best explored in murine models.

The exclusivity of T-bet in regulating human B cell immune responses to viruses versus other pathogens is an important question that has not been assessed. The expansion of cells resembling our T-bet^{high} population in other Th1 infections such as malaria suggests T-bet functions more broadly to promote humoral responses to intracellular pathogens (160). Additionally, IL-4 inhibits the T-bet transcriptional program in murine B cells, suggesting T-bet is not involved in Th2 B cell responses (116). Future work should assess T-bet induction and T-bet^{high} cell activation in the context of non-viral Th1 infections and also infections predicted to poorly elicit Th1 immunity, such as encapsulated bacteria or helminthes, to definitively define the pathogen responses T-bet regulates. Lastly, it should be noted that our findings suggesting an overwhelmingly T-bet-mediated humoral response to human viral infections do not rule out the involvement of T-bet⁻ subsets in aspects of the response.

T-bet^{high} B cells as a novel antiviral population

In another central finding of this study, we identified a novel T-bet⁺ viral infection-responsive subset of memory B cells in healthy individuals. A number of observations support the hypothesis that T-bet^{high} B cells are primed for acute viral infection responses: T-bet^{high} cells are enriched for IgG1 and IgG3 isotypes in healthy individuals, express a distinct trafficking receptor profile potentially facilitating tissue and inflammation-directed migration, demonstrate an activated phenotype and propensity to expand 2-4 weeks after infection with live replicating viral vaccines or HIV, expand and contract in concert with viremia during HIV infection, and dominate the HIV Env-specific B cell response in viremic HIV⁺ progressors. Many of T-bet^{high} cells' distinctive characteristics mirror those of effector T cells (100), suggesting T-bet^{high} cells are the specific B cell subset mediating the active humoral response to acute human viral infections (Figure 12).

Our study further suggests that T-bet^{high} cells are not a subset of classical memory B cells or antibody-secreting cells; instead, based upon their distinct transcriptional and phenotypic profiles and immune response kinetics compared to resting memory (RM) B cells and plasmablasts, they are likely a separate type of cell within the B cell lineage. This notion is supported by phylogenetic studies of CD21^{low} B cells that identified both distinct CD21^{low} clades and clonal relationships between these cells and the RM cell and plasmablast populations (204). T-bet^{high} cells' functional niche within the human humoral immune system is still unclear, but our transcriptional analyses identified intermediate expression of antibody-secreting program-promoting transcription factors (*IRF4*, *PRDM1*, and *XBP1*), suggesting these cells may be primed to quickly differentiate into antibody-secreting cells and contribute to the antibody response. Their distinctive trafficking receptor profile further suggests specialized migratory capacity, and murine studies suggest they may be capable of producing cytokines such as IFN γ upon stimulation (65). Future analyses should focus on elucidating the specific functions mediated by T-bet^{high} B cells during acute viral infections.

We also identified a unique tissue compartmentalization of T-bet^{high} cells: unlike plasmablasts and RM cells, which were identified in lymphoid tissues and can re-circulate back

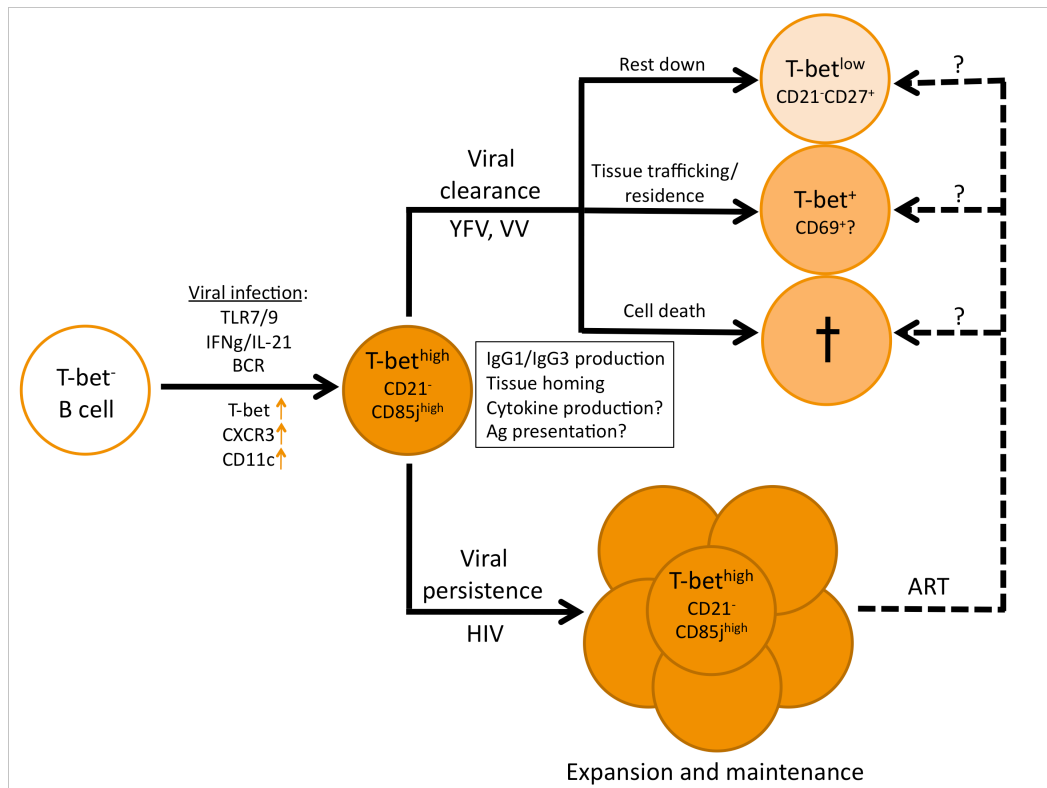


Figure 12: Model proposing the induction and differentiation of T-bet-expressing B cells by viral infections.

T-bet is induced in B cells by TLR/cytokine/BCR stimulation during acute viral infection, leading to T-bet^{high} cell development. Upon viral clearance (yellow fever virus (YFV) and vaccinia virus (VV) vaccinations), the blood T-bet^{high} subset contracts in size, potentially due to trafficking of cells into tissues, differentiation into a resting phenotype (T-bet^{low}), or cell death. However, during chronic HIV infection, persistent viral replication and immune activation induce expansion and maintenance of a large T-bet^{high} B cell pool that will return to normal size only upon control of viremia via antiretroviral therapy (ART).

into the blood, T-bet^{high} cells appear to be largely absent from lymphoid tissues (except for a curious T-bet^{high}CD21⁺ population in the spleen) and, based upon their absence from thoracic duct fluid, may not return back into the blood after they exit. Together, these observations suggest T-bet^{high} cells do not re-circulate in the absence of a significant immune response and raise questions regarding their trafficking and tissue localization. CD11c and CXCR3 expression would be predicted to enable tissue trafficking toward sites of inflammation, so T-bet^{high} cells may migrate from the blood directly into tissues where infection occurs. However, our data suggests T-bet^{high} cells do not return to the blood and instead may either reside permanently in these tissues or undergo apoptosis after completion of the acute immune response (Figure 12). Regardless, T-bet^{high} cells' tissue distribution and circulatory patterns indicate that lymphoid tissue immune surveillance (analogous to that of naïve and RM cells) is not a primary function of this subset.

Our findings are complemented and informed by two recent studies of early acute antigen-specific responses to infection and vaccination (159, 204). The first by Ellebedy et al. identified an activated, CD21^{low} B cell subset expressing CD71 that emerged acutely following influenza infection, ebola infection, and influenza vaccination, and contained the majority of influenza-specific B cells (159). While the second by Lau et al. did not detect a clear population expansion, the authors found a concentration of influenza-specific cells in the CD21^{low} population acutely following vaccination and an enrichment of T-bet expression in CD21^{low}- and RM-phenotype hemagglutinin-specific cells (204). In both cases, the cells' CD21^{low} phenotype and population dynamics during the acute immune responses suggest they are likely T-bet^{high} B cells. Together, these studies extend our findings of T-bet^{high} cell activation in primary YFV, VV, and HIV infections by implicating a broader involvement of T-bet^{high} cells in influenza and Ebola responses and suggesting T-bet^{high} cells can also be activated during secondary responses (influenza) and by non-replicating viruses (inactivated influenza vaccine).

T-bet^{low} B cells as a quiescent memory population

In addition to Tbet^{high} cells, our study identified a counterpart T-bet^{low} subset within the RM B cell population. As their name suggests, T-bet^{low} cells express T-bet at a low MFI that is difficult to confidently detect using most intracellular cytokine staining protocols. This population differs significantly from Tbet^{high} cells in a number of other phenotypes and behaviors, including consistent CD21 and CD27 expression, minimal signs of activation, an unresponsiveness to acute viral infections in terms of activation and expansion (at the population level), and a widespread lymphoid tissue distribution with the ability to re-circulate back into the blood. These characteristics are largely consistent with a resting, long-lived memory B cell phenotype (151); as such, we speculate that T-bet^{low} cells represent a reservoir of virus-specific clones that function to maintain long-lived humoral memory to viral infections and facilitate rapid responses upon antigen re-encounter. The existence of a human T-bet^{low} memory population is supported by mouse models in which IgG2a⁺ memory B cells require consistent low-level T-bet expression to maintain their integrity well after class-switching has completed (112).

If T-bet^{low} cells are indeed a resting, virus-specific reservoir, T-bet^{high} cell clones generated early during acute viral infection would be predicted to seed the T-bet^{low} population upon resolution in the contraction phase (Figure 12). In support of this phenomenon, influenza-specific, acutely activated CD21^{low} clones have been demonstrated to enter the long-lived memory pool (159), and common origins of pathogen-specific TLM (CD21⁺CD27⁺; almost certainly T-bet^{high} cells based on our findings) B cells and RM cells have been described during HIV infections (187). In our own study, the T-bet^{low} population increasingly maintained the Env-specific memory B cell pool upon control of viremia and subsequent contraction of the T-bet^{high} subset, and T-bet^{low} cells' maintenance in the absence of detectable HIV viremia suggests an ability to survive independent of antigen and significant inflammatory signals.

Plasmablasts can express T-bet

Previously, Wang et al. described low-level T-bet mRNA expression in plasmablasts during a hapten response in mice (112). To our knowledge, ours was the first study to identify T-bet protein expression in plasmablasts. T-bet was essentially undetectable in the plasmablasts of healthy individuals in the absence of an immune response; however, YFV- and VV-vaccinated individuals reproducibly exhibited a significant spike in plasmablast T-bet MFI that peaked around 2 weeks post vaccination but remained lower than the MFI in T-bet⁺ memory B cells. T-bet MFI in plasmablasts also increased during acute HIV infection and was maintained at an elevated MFI in chronically infected individuals, suggesting T-bet induction may contribute to the IgG1-focused hypergammaglobulinemia characteristic of viremic HIV infection. T-bet's function in plasmablasts is unclear, but it may be required for the continuous transcription of IgG1 and IgG3 that would occur in cells constitutively producing antibody (112). T-bet may also help plasmablasts traffic to sites of inflammation by promoting CXCR3 expression.

Human spleen is a T-bet⁺ B cell-rich organ

Our identification of a massive CD21⁺T-bet⁺ B cell population in human spleen was particularly surprising since the magnitude more closely resembled reactive lymph nodes than the non-reactive nodes assessed in our study (123). It remains possible that this phenomenon is the result of unrecognized inflammation in our spleen samples, but the consistently large CD21⁺T-bet⁺ population in all six donors suggests this is a common phenotype. Our panel was insufficient to determine the identity of these cells and their relationship to peripheral T-bet⁺ subsets, but they were chiefly comprised of cells with marginal zone B cell-like (CD21⁺CD27⁺IgM⁺IgD⁺) and class-switched memory-like (CD21⁺CD27⁺IgM⁻IgD⁻) phenotypes. Interestingly, we also detected T-bet expression in phenotypically naïve cells (CD21⁺CD27⁻IgD⁺), suggesting the spleen may be a site of T-bet⁺ B cell activation. The considerable population size of splenic CD21⁺T-bet⁺ B cells suggests a portion of these cells may be spleen-resident, raising the possibility that human spleen acts as a reservoir of T-bet⁺ B cells. Histological, transcriptional, and phylogenetic studies

are necessary to identify the localization of these cells (white pulp, red pulp, or marginal zone) and determine their relationship to peripheral blood T-bet⁺ B cells.

Tissue-like memory B cells are not inherently dysfunctional

Our data identify an association between T-bet^{high} B cell induction and protective vaccine-induced antibody responses, and this connection is further supported by influenza studies (159, 204) and T-bet⁺ B cells' clear association with control of viral load in mice (117, 118). Together, these studies suggest T-bet^{high} cells are not a functionally impaired developmental aberration and are instead a normal component of acute humoral responses. This notion has implications for the HIV-specific B cell response (discussed below), but it also contradicts a prevalent conviction that tissue like memory (TLM) cells, a large component of the T-bet^{high} population, are inherently dysfunctional (151). Expansion of this normally rare population during chronic infectious diseases (120, 151, 160) and autoimmune diseases (73, 119, 162, 163) is generally interpreted as evidence of an aberrant differentiation state, and in some cases such as SLE, these cells do contribute to pathology (119). However, in the context of HIV infection, our findings suggest that T-bet^{high} (and TLM) cell expansion is a product of uncontrolled viral replication and inflammation continuously directing newly activated B cells along the T-bet^{high} differentiation pathway. Since T-bet^{high} population size returns to normal upon control of viremia, we propose that HIV is simply driving the consistent development of a normally transient population. Importantly, our findings do not rule out the possibility that T-bet^{high} B cells with a TLM phenotype effectively participate in acute viral responses but specifically undergo exhaustion and become dysfunctional if they are abnormally maintained for an extended period of time in the presence of high antigen load (205). Functional studies comparing T-bet^{high} cells in HIV⁻ and HIV⁺ individuals are necessary to definitively rule out this possibility, but our phenotypic and transcriptional comparisons in these cohorts suggest T-bet^{high} cells themselves are largely unchanged by chronic infection.

Several TLM cell characteristics interpreted to reflect dysfunction or anergy can be explained by the biology of the T-bet^{high} B cell subset. First, TLM cells poorly respond to

traditional BCR-based stimulation *in vitro* (151). This property has been confirmed in murine models, which suggest T-bet⁺ B cells are a subset with distinct requirements for activation. BCR/CD40 stimulation poorly activates mouse T-bet⁺ B cells, while BCR and concomitant TLR7 and TLR9 stimulation potently drive activation of this subset (114, 115). Analyses of human TLM B cells have largely failed to assess these combinations known to efficiently activate T-bet⁺ B cells and may therefore misrepresent the cells' capacity for responsiveness. In combination with IFN γ and IL-21, TLR7 and TLR9 stimulation also efficiently drive T-bet⁺ B cell differentiation from precursors (116), suggesting HIV infection likely induces T-bet in human B cells by directly providing TLR7 and TLR9 ligands (206-210) and indirectly stimulating production of Th1-associated cytokines (IL-12, IL-15, IL-18, TNF, and IFN γ) during both acute and chronic phases of infection (211-213).

Second, TLM cell-derived HIV Env-specific antibodies have been described as inferior, demonstrating decreased somatic hypermutation and HIV neutralization capacity compared to resting memory B cells (187). In line with this observation, analyses of human CD27⁺IgG⁺ B cells (a large component of the TLM population) and CD21^{low} cells strongly suggest T-bet^{high} (and TLM) cells are relatively recent germinal center emigrants and, as such, would be expected on a population level to demonstrate reduced affinity maturation compared to the mature resting memory pool (72, 73, 204). It remains possible that TLM-phenotype cells exhibit HIV infection-specific antibody maturation deficits due to impaired B cell help, but antibodies derived from *Plasmodium falciparum*-specific TLM B cells are highly mutated and neutralize comparably to antigen-specific resting memory B cell counterpart (188), suggesting diminished antibody mutation is not necessarily characteristic of TLM-phenotype cells.

Lastly, with regard to the aforementioned points, it becomes difficult to extrapolate from TLM B cell findings and relate these to T-bet^{high} cells as most TLM B cell studies are complicated by CD21⁺CD27⁺ population heterogeneity: we and others identified significant differences between the T-bet⁺ and T-bet⁻ subsets that together comprise the TLM B cell population (171), but studies have generally assessed the TLM population as a whole. Accordingly, future analyses should

specifically focus on the characteristics of the T-bet⁺ TLM B cell population to understand the role of TLM B cells during infection.

T-bet⁺ B cells dominate the memory B cell response to HIV Env

Our surprising identification of T-bet⁺ B cells as mediators of the HIV Env response is a critical finding that generates a number of questions and holds implications for HIV immunity and vaccine efforts. This observation definitively confirms T-bet⁺ B cells as mediators of humoral antiviral responses, a concept that was implicated by our YFV and VV response data, and further suggests T-bet induction as the mechanism promoting an IgG1-focused Env-specific antibody response in HIV-infected individuals. An important question remaining is the degree to which expanded T-bet⁺ B cells in infected individuals are virus-specific, as our HIV gp140 probe cannot capture all Env-specific cells and B cell responses generated against other HIV proteins such as Gag were not assessed. HIV infection is characterized by polyclonal B cell activation (147), and the excessive magnitude of T-bet induction suggests bystander activation may contribute to the expanded T-bet⁺ B cell population, but our findings indicate that the T-bet-regulated response is at least partially virus-specific.

As T-bet induction in B cells is critical for control of murine viral infections and associates with protective vaccine responses in humans, we originally hypothesized that an inability to mount a T-bet⁺ B cell response might partially explain the shortcomings of humoral immunity during HIV infection. However, our identification of a Th1-biased, T-bet-regulated B cell and antibody response in both viremic and controlling individuals suggests the humoral immune system mounts an appropriately focused B cell response to HIV Env, even in settings of immunopathology. While B cell development does not seem to be misdirected by HIV per se, a number of other HIV infection-related factors may impede the T-bet-regulated response and prevent timely development of broad neutralization, including loss of CD4⁺ T cell help, disruption of lymphoid tissue architecture, and the difficult antigenic nature of the virus.

Humoral immunity (and T-bet⁺ B cells by extension) cannot effectively neutralize and clear HIV in infected individuals. However, as antibodies can put significant pressure on the virus (138, 140, 214), an important question remaining is whether T-bet⁺ B cells can still contribute to the HIV immune response. T-bet⁺ B cells and their antibodies are critical for maintaining low level chronic viremia in mouse models (117, 118), and several human and macaque B cell depletion studies demonstrate a loss of HIV/SIV set point control upon B cell and neutralizing antibody titer decline (198, 200, 202), together suggesting that the T-bet⁺ B cell-mediated antibody response may contribute to the maintenance of set point viremia in some cases. A tractable model would be necessary to decisively determine this, but human studies assessing the relationship between T-bet induction and immunological or disease outcomes may provide insight into T-bet's potential role in the immune response (e.g. does an earlier B cell T-bet response or one of greater magnitude correlate with viral set point or development of neutralization breadth?). In any case, our findings suggest that any humoral immunity-based pressure on the virus is likely derived from T-bet⁺ B cells and the HIV Env antibody response they regulate.

Considerations for vaccines

Perhaps most importantly, our findings identify both T-bet itself and T-bet⁺ B cells (particularly the T-bet^{high} subset) as targets of interest for vaccines and therapeutic interventions attempting to induce or manipulate Th1-focused humoral immunity. Similarly, T-bet induction in B cells could serve as a reliable biomarker to assess vaccine candidates' ability to drive Th1 B cell programs. Recent immune correlates analyses of the RV144 HIV vaccine trial identified an association between the elicitation of IgG3-isotype antibodies and reduced infection risk (215, 216), suggesting T-bet induction may be directly relevant to future vaccine efforts attempting to consistently recreate this response. TLR7/8 and TLR9 adjuvants may hold promise for promoting T-bet-regulated vaccine responses, and a number of studies show that these adjuvants significantly improve the quality of antibodies generated by vaccination (217, 218). In summary, a

more detailed understanding of T-bet⁺ B cell biology will be informative for the development of interventions to improve humoral immunity to viruses.

CHAPTER 5

MATERIALS AND METHODS

Study participants:

Acute infection studies: Five healthy, HIV-negative individuals received live-attenuated YFV-17D vaccine (YF-Vax, Sanofi Pasteur) and seven different healthy, HIV-negative individuals received live vaccinia smallpox vaccine (Dryvax, Wyeth Laboratories). Peripheral blood mononuclear cell (PBMC) samples were obtained on or near day of vaccination and at several subsequent time points (see Table 1 for donor information). Seven acutely infected HIV⁺ donors were enrolled in the RV217 Early Capture HIV Cohort run by the US Military HIV Research Program. PBMC sample timing was calculated relative to first positive HIV RNA test (Abbot Real-Time HIV-1 assay, Abbot Laboratories, Abbott Park, IL). Samples included a pre-infection time point (ranging 478-41 days previous to first positive HIV RNA test.), an early acute infection time point (ranging 1-21 days after first positive test; these were collected during or shortly after peak acute viremia and are considered to represent an early acute infection time point), and a chronic infection time point (see Table 2). Individuals were antiretroviral therapy-naïve at all sample time points assessed.

Tissue studies: Paired blood and thoracic duct fluid samples were obtained from individuals with idiopathic or trauma-based chylopericardium or chylothorax requiring intervention at the Hospital of the University of Pennsylvania. Lymphoid tissue samples (mesenteric lymph node, iliac lymph node, tonsil, and spleen) were obtained at the Hospital of the University of Pennsylvania and Case Western Reserve University: mesenteric and iliac lymph nodes were obtained as normal structures during abdominal surgery and kidney transplant surgery, respectively. Non-enlarged tonsils were obtained from sleep apnea patients. Spleens were removed and obtained due to

trauma or surgical intervention. Mononuclear cells were mechanically separated from solid tissues and enriched using a ficoll gradient.

Chronic HIV infection studies: PBMCs and serum from ART-naïve, viremic HIV⁺ individuals (referred to as progressors) and PBMC from ART-treated, aviremic HIV⁺ individuals (referred to as ART) were obtained from the University of Pennsylvania Center for AIDS Research (CFAR; see Supplementary Tables 1 and 3). HIV-negative serum samples age- and ethnicity-matched to the ART-naïve, viremic HIV⁺ individuals were obtained from Case Western Reserve University. PBMCs from viremic controllers and elite controllers (VC and EC, respectively) were obtained from individuals enrolled in the SCOPE study at the University of California San Francisco (see Supplemental Table 2). Additional progressor PBMCs were obtained from the University of Toronto (see Supplemental Table 3). Healthy HIV-negative human donor PBMC samples were obtained from the University of Pennsylvania Human Immunology Core.

Antibody reagents:

The following antibodies were obtained from Biolegend: CD19 BV785 (clone HIB19), CD3 APC-Cy7 and BV570 (clone UCHT1), IgM BV605 (clone MHM-88), CD21 PECy7 (clone Bu32), CD27 BV650 (clone O323), CD38 BV421 and BV711 (clone HIT2), CXCR3 BV711 and APC (clone G025H7), CD86 BV605 (clone IT2.2), CD10 BV605 (clone HI10a), CD71 FITC (clone CY1G4), PD-1 BV421 (clone EH12.2H7), CD8 BV711 (clone RPA-T8), CD56 BV605 (clone 5.1H11), CD16 PECy5 (clone 3G8). The following antibodies were obtained from BD Biosciences: CD14 APC-Cy7 (clone MØP9), CD16 APC-Cy7 (clone 3G8), IgD PECF594 and AF700 (clone IA6-2), IgG AF700 (clone G18-145), CD85j FITC (clone GHI/75), Ki67 AF700 and FITC (clone B56), CD21 BV421 (B-ly4), CD95 PECy5 (clone DX2), Bcl-6 PE (clone K112-91). The following antibodies were obtained from eBiosciences: T-bet PE and PECy7 (clone eBio4B10), CD10 PECy5 (clone eBioCB-CALLA), CD11c PECy5.5 (clone 3.9), CD85j APC (clone HP-F1), CD7 PECy7 (clone 124-1D1), Eomesodermin AF647 (clone WD1928). CD69 PECy5

(clone TP1.55.3) was obtained from Beckman Coulter. The following antibodies were obtained from Southern Biotech: IgG1 PE (clone HP6001), IgG2 AF488 (clone HP6002), IgG3 AF647 (clone HP6050). IgA FITC (polyclonal) and CD4 PECy5.5 (clone S3.5) were obtained from Invitrogen. FcRL4/5 antibody (clone 2A6) was gifted by Max D. Cooper (Emory University, Atlanta); this antibody was initially characterized as FcRL4-specific, but a recent study demonstrated clone 2A6 reacts with both FcRL4 and FcRL5 (56).

Antibody staining, flow cytometry acquisition, and analysis:

Cryopreserved PBMCs (or mononuclear cells from other tissues) were thawed, counted, examined for viability, and rested overnight at 37°C and 5% CO₂ in RPMI with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin added. All incubations during the following staining process were performed at room temperature in the dark. On the following morning, PBMCs were washed with PBS and stained for viability with aqua amine-reactive viability dye (Invitrogen) for 10 minutes. Cells were stained with a prepared cocktail of surface antibodies for 25 minutes, washed once with PBS plus 0.1% sodium azide and 1% BSA, and fixed/permeabilized using the eBiosciences FoxP3 Transcription Factor buffer kit (cat# 00-5523-00). Intracellular targets were stained with a separately prepared cocktail of antibodies for 60 minutes, washed with the eBioscience kit buffer, fixed with PBS plus 1% paraformaldehyde, and stored in the dark at 4°C until acquisition. RV217 cohort samples (Figures 2G, 2H) were stained identically except BD Cytofix/Cytoperm Kit (Cat. # 554722) was used to fix/permeabilize cells for intracellular staining. Compensation controls were produced with antibody capture beads (BD Biosciences) for each antibody and ArC Amine Reactive beads (ThermoFischer Scientific) for aqua amine-reactive dye. All flow cytometry data was collected on a modified LSR II (BD Immunocytometry Systems). Data was analyzed using FlowJo software (TreeStar). Graphs were created and statistical analysis was performed using GraphPad Prism (version 7.0a).

HIV gp140 probe staining:

Recombinant group M consensus gp140 protein was produced and conjugated to Brilliant Violet 421 and Alexa Fluor 647 fluorophores, as previously described (152, 219). Washed PBMCs were pre-treated with anti-CD4 (clone SK3, Biolegend) previous to aqua amine-reactive dye staining to prevent interactions between gp140 probe and CD4. gp140 probe was then added to surface marker cocktail and staining procedure was completed as described above.

Serum antibody characterization:

HIV-1 specific and total IgG1- IgG4, IgA and IgM were measured on a Bio-Plex instrument (Bio-Rad) as previously described (136, 215, 220). gp140-specific antibodies were captured using the same group M consensus sequence protein from gp140-specific cell staining (see above).

Cell sorting and Fluidigm Biomark transcript analysis:

PBMCs from HIV-infected and –uninfected patients were thawed and rested overnight. After being washed in PBS, the cells were incubated with amine-reactive viability dye for 10 minutes and stained with an undiluted cocktail of surface antibodies at room temperature. Cells were washed and resuspended in R10. One hundred cells each from different B cell populations were sorted on a FACS Aria II (BD Biosciences) into individual wells of a 96-well PCR plate according to the gating strategy depicted in supplemental figure 1A. Each well contained 5 μ L lysing buffer, consisting of 4.725 μ L of DNA Suspension Buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA; TEKnova), 0.025 μ L of 20 U/ μ L SUPERase•In™ (Ambion) and 0.25 μ L of 10% NP40 (Thermo Scientific). After FACS sorting, PCR plates were frozen and kept in -80°C until usage. PCR plates were thawed on ice and pre-heated for 90 seconds at 65°C. Subsequently, 1 μ L Reverse Transcription Master Mix (Fluidigm) was added to each well and placed into a thermocycler for reverse transcription (25°C for 5 min, 42°C for 30 min, 85°C for 5 min). Next, 4 μ L of a pre-amplification mix, consisting of 1 μ L pooled mixture of all primer assays (500nM), 2 μ L

5× PreAmp Master Mix (Fluidigm) and 1 µL H₂O, was added to each well and run on a thermocycler (95°C for 5 min followed by 18 cycles: 96°C for 5 sec 60°C for 6 min). To remove excess primers, 4 µL of an Exonuclease mixture, containing 0.8 µL 20 units/µL of Exonuclease I (New England BioLabs), 0.4 µL 10× Exonuclease I Reaction Buffer (New England BioLabs), and 2.8 µL H₂O was added to each well. The plate was run on a thermocycler (37°C for 30 min, 80°C for 15 min). Each well was then diluted (1:4) with DNA Suspension Buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA; TEKnova). In a new PCR plate, distinct primer assays were generated by adding individual primer pairs (5µM) together with a mix of 2.6 µL 2x Assay Loading Reagent (Fluidigm) and 2.4 µL 1x DNA suspension buffer to each well. Similarly, a sample PCR plate was created by dispensing 4 µL of a sample master mix containing 3.5 µL 2x Sso Fast EvaGreen Supermix with Low ROX (Bio-Rad), 0.35 µL 20x DNA Binding Dye Sample Loading Reagent (Fluidigm) and 0.15 µL H₂O to each well. An additional 3 µL of pre-amplified samples were added to each well. Control line fluid (Fluidigm) was injected to the 96.96 Dynamic Array chip (Fluidigm) and the chip was primed using an IFX Controller HX. After priming the chip, 4.5 µL of the primer assays and 5 µL of the sample mix were added to detector inlets of the chip and transferred to the IFX Controller HX for loading the mixtures. The chip was then transferred to a Biomark HD instrument (Fluidigm) and run using the GE Fast 96x96 PCR+Melt v2.pcl program. All primers were purchased from IDT and assay efficiency as well as melting and amplification curves for each assay were evaluated beforehand on a separate Biomark HD run with similar sorted B cell populations. All data were pre-analyzed with the Real-time PCR analysis software (Fluidigm), and Linear (Derivative) and User (Detectors) were used as settings to generate Ct values. A conservative Ct value of 25 was used as limit of detection (LOD). Relative gene expression was defined as a log₂ value based on: $\log_2 = \text{LOD} - \text{Ct}$. All subsequent analysis of the gene expression data, including tSNE analysis and hierarchical clustering, were performed using R Studio.

T-bet siRNA knockdown:

Mature CD21⁺CD27⁺ B cells from viremic HIV⁺ donors were transfected with 500 nM of control non-targeting or *TBX21*-specific On-target plus smart pool siRNAs (Thermo Fisher Scientific) using the Lonza nucleofection 96-well plate system, according to manufacturer specifications and as previously described (178). Cells were rapidly transferred to preheated complete medium (RPMI 1640-10% FBS) and incubated for 24 hours at 37°C. Cell viability was evaluated by vital dye exclusion (Guava Technologies). *TBX21* knockdown efficiency and impact on *AICDA* expression were evaluated by quantitative RT-PCR, as previously described (178). Briefly, total RNA was extracted using the RNeasy micro kit with on-column DNA digestion (Qiagen), according to manufacturer specifications. Total RNA was reverse transcribed and analyzed using TaqMan probe/primer mix (Applied Biosystems) by one-step quantitative PCR (Applied Biosystems 7500 system). Data were normalized to the housekeeping gene *POLR1A* by a comparative $\Delta\Delta C_t$ method.

Statistics:

Paired or unpaired t-tests (two-tailed) were used to compare two groups of data. When three or more groups were compared, one way ANOVA or repeated measures one way ANOVA, each combined with Tukey's multiple comparisons test, were used. Correlative relationships were assessed using Spearman correlation. Error bars on all plots represent mean \pm SEM. A P value $P < 0.05$ was considered significant.

Study approval:

Written informed consent was obtained from study participants and blood/tissue samples were collected with institutional review board approval at each collecting institution: Oregon Health and Science University (IRB# 2470, IRB# 2832), University of Pennsylvania (IRB#809316, IRB# 815056), Case Western University (IRB# 10-09-12), University of Toronto (REB# 12-378), University of California San Francisco (IRB# 10-01330), Human Subjects Protection Branch

(RV217/WRAIR#1373), The United Republic of Tanzania Ministry of Health and Social Welfare (MRH/R.10/18/VOLL.VI/85), Tanzanian National Institute for Medical Research (NIMR/HQ/R.8aVol.1/2013), Royal Thai Army Medical Department (IRBRTA 1810/2558), Uganda National Council for Science and Technology– National HIV/AIDS Research Committee (ARC 084), and Uganda National Council of Science and Technology (HS 688). This work was conducted in accordance with the principles expressed in the Declaration of Helsinki.

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